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(54) Title: CONJUGATES OF A POLYMER AND A PROTEIN LINKED BY AN OXIME LINKING GROUP

(57) Abstract: The present invention relates to polymers functionalized by an aminooxy group or a derivative thereof, conjugates, wherein the functionalized polymers are covalently coupled with a protein by an oxime linking group, a process for preparing the functionalized polymers, a process for preparing the conjugates, functionalized polymers as obtainable by the process of the present invention, conjugates as obtainable by the process of the present invention, and pharmaceutical compositions comprising at least one conjugate of the present invention and the use of said conjugates and compositions for the prophylaxis or therapy of the human or animal body.



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Conjugates of a Polymer and a Protein
linked by an Oxime Linking Group

The present invention relates to polymers functionalized by an aminooxy group or a derivative thereof, conjugates, wherein the functionalized polymers are covalently coupled with a protein by an oxime linking group, a process for preparing the functionalized polymers, a process for preparing the conjugates, functionalized polymers as obtainable by the process of the present invention, conjugates as obtainable by the process of the present invention, and pharmaceutical compositions comprising at least one conjugate of the present invention and the use of said conjugates and compositions for the prophylaxis or therapy of the human or animal body.

Covalent attachment of functionalized polymers like polysaccharides, for example starch and derivatives thereof, and dextrane and derivatives thereof and polyethylene glycol and derivatives thereof to therapeutic proteins prolongs the circulatory life time of the proteins *in vivo*, reduces their antigenicity and immunogenicity, and improves their resistance to proteolysis. These properties are of great clinical interest, especially in the case of relatively small proteins, where it is believed that an increase of Stoke's radius is consistent with a reduced renal clearance.

Modifications of proteins with biocompatible polymers are known in the art. So far most of the modifications of proteins have been carried out by using polyethylene glycol or dextran, whereby polyethylene glycol (PEG) has been preferably used. Further, modifications of proteins with other polysaccharides are known in the art.

WO 02/09766 discloses, among others, biocompatible protein-polymer compounds which are produced by conjugation of biologically active protein with a biocompatible polymer derivative. The biocompatible polymers used are highly reactive branched polymers, and the resulting conjugates contain a long linker between polymer derivative and protein. As biocompatible polymers, polymers of formula $(P-OCH_2CO-NH-CHR-CO-)_n-L-Qk-A$ are

described, wherein P and Q are polymeric residues and k may be 1 or 0. For P and Q, polyethylene glycol, polypropylene glycol, polyoxyethylene, polytrimethylene glycol, polylactic acid and its derivatives, polyacrylic acid and its derivatives, polyamino acid, polyvinyl alcohol, polyurethane, polyphosphazene, poly(L-lysine), polyalkylene oxide, polyacryl amide and water soluble polymers such as dextran or polysaccharide are mentioned. As proteins, among others, alpha, beta and gamma interferons, blood factors, cytokines such as interleukins, G-CSF, GM-CSF are mentioned. In the examples of WO 02/09766, only mono-, di- and tri-polyethyleneglycol derivatives are disclosed which are coupled exclusively to interferon and epidermal growth factor, and human growth hormone.

WO 94/01483 discloses biocompatible polymer conjugates which are formed by covalently binding a biologically inactive polymer or polymer derivative to a pharmaceutically pure, synthetic hydrophilic polymer via specific types of chemical bonds. As naturally occurring polymers and derivatives thereof, polysaccharides such as hyaluronic acid, proteoglycans such as chondroitin sulfates A, B and C, chitin, heparin, heparin sulfate, dextrans such as cyclodextrane, hydroxyethyl cellulose, cellulose ether and starch, lipids such as triglycerides and phospholipids are disclosed. As synthetic polymers, among others, polyethylene and derivatives thereof are described having an average molecular weight of from about 100 to about 100,000. As proteins linked to the polymer or the polymer derivative, cytokines and growth factors are described, including interferons, tumor necrosis factors, interleukins, colony stimulating factors, growth factors such as osteogenic factor extract, epidermal growth factor, transforming growth factor, platelet derived growth factor, acidic fibroblast growth factor and others are disclosed. In all working examples of WO 94/01483, polyethylene glycols derivatives are used as polymer.

WO 96/11953 discloses N-terminally chemically modified protein compounds and methods of their production. Specifically, G-CSF compositions are described which result from coupling a water soluble polymer to the N terminus of G-CSF. In the context of WO 96/11953, also consensus interferone N-terminally coupled to water soluble polymers are disclosed. While a wide variety of water soluble polymers are listed in WO 96/11953 (e.g. copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), poly(n-vinyl pyrrolidone)polyethylene glycol, polypropylene glycol

homopolymers, polypropylene oxide/ethylene oxide copolymers or polyoxyethylated polyols), only PEGylated G-CSF or consensus IFN compositions are described in the working examples of WO 96/11953.

5 WO 97/30148 relates to polypeptide conjugates with reduced allergenicity comprising a polymeric carrier molecule having two or more polypeptide molecules coupled thereto. These conjugates are preferably part of compositions used in the personal care market. Said conjugates are produced by activating a polymeric carrier molecule, reacting two or more polypeptide molecules with the activated polymeric carrier molecule and blocking of residual
10 active groups on the conjugate. As polymeric carrier molecule, a vast variety is listed in WO 97/30148, including such different groups of compounds like natural or synthetic homopolymers such as polyols, polyamines, polycarboxylic acids and heteropolymers comprising at least two different attachment groups. Examples are given, which comprise star PEGs, branched PEGs, polyvinyl alcohols, polycarboxylates, polyvinylpyrrolidones and poly-
15 D,L-amino acids. Among others, also dextrans such as carboxymethyl dextran, celluloses such as hydroxyethyl cellulose or hydroxypropyl cellulose, hydrolysates of chitosan, starches such as hydroxyethyl starches or hydroxypropyl starches, glycogen, agarose, guar gum, inulin, pullulan, xanthan gum, carrageenin, pectin, alginic acid etc. are disclosed. As polypeptides, only some enzymes are explicitly disclosed.

20

Baldwin, J.E. et al., Tetrahedron, vol. 27 (1981), pp. 1723 - 1726 describe the chemical modification of dextran and hydroxyethyl starch to give aldehyde substituted polymers which are allowed to react with hemoglobin to give soluble polymer-bound hemoglobins. These were shown to be capable of binding oxygen, but heart perfusion experiments clearly
25 indicated that the polymer-bound hemoglobins were not suitable for use as blood substitutes.

WO 99/49897 describes conjugates of hemoglobin formed by reacting polysaccharides such as dextrane or hydroxyethyl starch with amino groups of the hemoglobin. As functional groups of the polysaccharide, aldehyde groups produced by oxidative saccharide ring-opening
30 are used. As preferred reducing agent used, borane dimethylamine is disclosed. Moreover, WO 99/49897 is exclusively limited to hemoglobin.

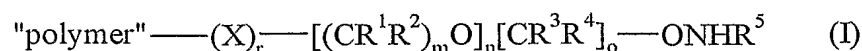
WO 03/074087 relates to a method of coupling proteins to a starch-derived modified polysaccharide. The binding action between the protein and the polysaccharide, hydroxyalkyl

starch, is a covalent linkage which is formed between the terminal aldehyde group or a functional group resulting from chemical modification of said terminal aldehyde group of the hydroxy alkyl starch molecule, and a functional group of the protein. As reactive group of the protein, amino groups, thio groups and carboxyl groups are disclosed, and aldehyde groups of the protein are not mentioned. Moreover, while a vast variety of possibilities of different linkages is given in the form of many lists, including different functional groups, theoretically suitable different linker molecules, and different chemical procedures, the working examples describe only two alternatives: first, an oxidized hydroxyethyl starch is used and coupled directly to proteins using ethyldimethylaminopropyl carbodiimide (EDC) activation, or a non-oxidized hydroxyethyl starch is used and coupled directly to a protein forming a Schiff's base which is subsequently reduced to the respective amine.

Gaertner et al., Bioconjugate Chem. 1996, 7, 38-44 discloses a site specific attachment of functionalized polyethylene glycol (PEG) to the amino terminus of proteins. The PEG and the protein are linked by an -NHCO-CH₂-O-N=CH- linking group.

It is an object of the present invention to provide conjugates of a functionalized polymer which is covalently linked with a protein to improve the circulatory life time *in vivo* of the proteins employed and to reduce the antigenicity and immunogenicity of the proteins.

This object is achieved by a functionalized polymer of formula I



wherein the symbols have the following meanings

"polymer" soluble linear or branched homopolymers or random copolymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, preferably ethylene glycol homopolymers (PEG), propylene glycol homopolymers, alkylene glycol copolymers, preferably propylene oxide/ethylene oxide co-polymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids, and polysaccharides, preferably selected from the group consisting of starch, cellulose, dextran, gum arabic, xanthan gum, inulin, ghatti

gum, pectin, guar gum, gum tragacanth, agar, algin, karaya gum, carrageenan, scleroglucan, fucellaran, arabinogalacton and locust bean gum;

$R^1, R^2, R^3,$

5 R^4, R^5 hydrogen, alkyl, aryl, more preferably hydrogen;

m 2 to 4, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2 ;

n 0 to 20, preferably 0 to 10, more preferably 1 to 5, most preferably 2;

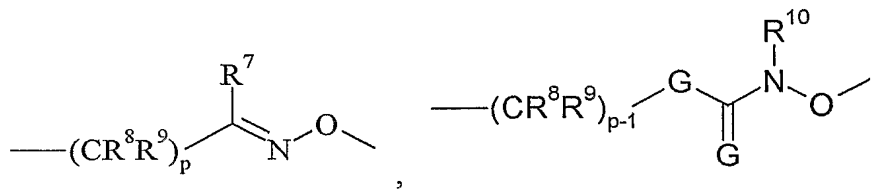
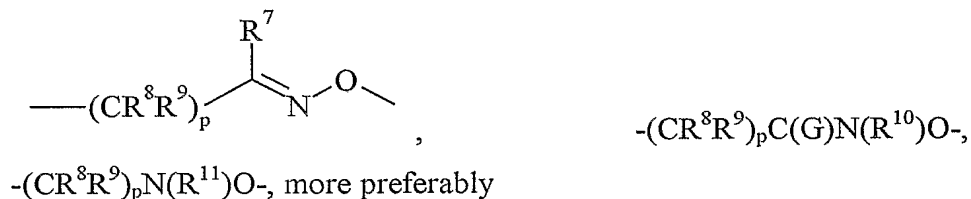
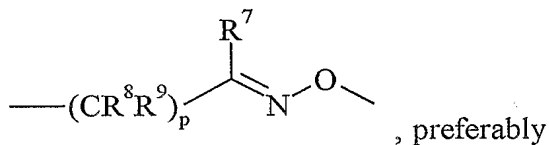
o 0 to 20, preferably 0 to 10, more preferably 0 or 2, wherein in the case of n =

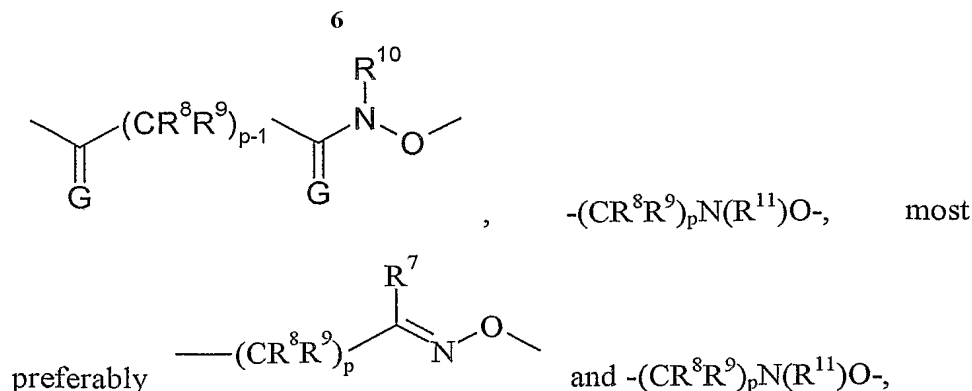
10 0, o is not 0,

in a preferred embodiment o is 2 to 20, preferably 2 to 10, more preferably 2, wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4 ;

r 0 or 1, preferably 1;

15 X $-(CR^8R^9)_pO-$, $-(CR^8R^9)_pS-$, $-(CR^8R^9)_pNR^6-$, $-(CR^8R^9)_pOC(O)-$, $-(CR^8R^9)_pC(O)O-$, $-(CR^8R^9)_pC(G)N(R^{10})O-$, $-(CR^8R^9)_pN(R^{11})O-$,





wherein one or more groups $\text{---}(\text{CR}^8\text{R}^9)\text{---}$ may be replaced by W, whereby a chemically reasonable group is formed;

W O, NR^{12} , C(G), preferably O, C(G);

G S, O, NR^{14} , preferably O;

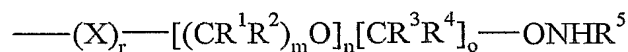
$\text{R}^6, \text{R}^7, \text{R}^8,$

$\text{R}^9, \text{R}^{10},$

$\text{R}^{11}, \text{R}^{12}, \text{R}^{14}$ hydrogen, alkyl, aryl, preferably hydrogen,

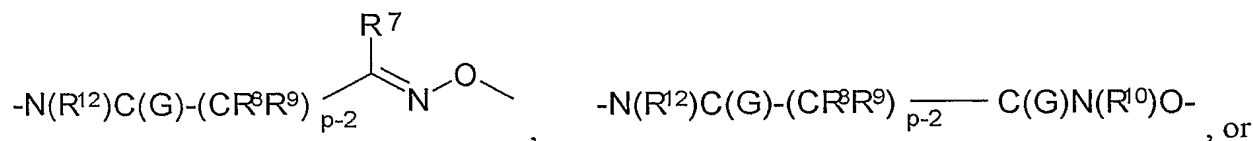
p 0 to 20, preferably 0 to 10, more preferably 0 to 5, most preferably 0 to 4, even more preferably 0 in the case of polysaccharides and derivatives thereof, and 1 to 4 in the case of polyalkylene glycols and derivatives thereof, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 ;

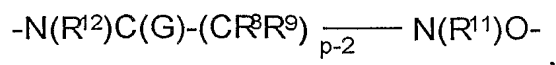
wherein the group



is covalently linked with least one terminal group or least one centrally located group of the "polymer".

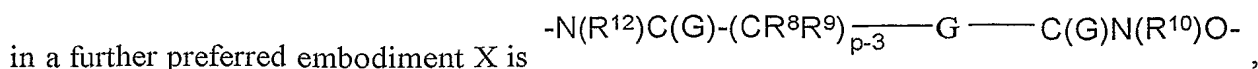
With regard to above-mentioned group X, preferably in the case of polyalkylene glycols and derivatives thereof, two of the groups $\text{---}(\text{CR}^8\text{R}^9)\text{---}$ may be replaced by two groups W, preferably by two groups W such that the two groups W together form a group $\text{---N(R}^{12})\text{C(G)---}$. In this case, X is more preferably





wherein p is 2, 3 or 4, preferably 3 or 4 and most preferably 4, with

$R^7, R^8, R^9, R^{10}, R^{11}, R^{12}$ hydrogen, alkyl, aryl, preferably hydrogen,

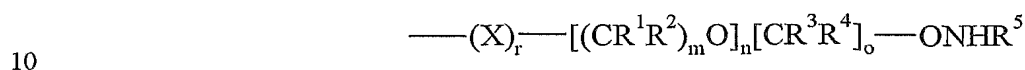


in a further preferred embodiment X is

5 wherein p is 5, with

$R^7, R^8, R^9, R^{10}, R^{11}, R^{12}$ hydrogen, alkyl, aryl, preferably hydrogen.

With regard to above-mentioned group X, preferably in the case of polyalkylene glycols and derivatives thereof, also structures



are preferred where $r = 1$ and $X = -(CR^8R^9)_pO-$, $-N(R^{12})C(G)-(CR^8R^9)_{p-2}-S-$ or $-(CR^8R^9)_pS-$, wherein in the case of $-N(R^{12})C(G)-(CR^8R^9)_{p-2}-S-$ p is 2 to 20, preferably 2 to 10, more preferably 2 to 5, most preferably 2 to 4. In these cases, it is still more preferred that $n = 0$ and $o = 2$ to 10, preferably 2 to 8 and still more preferably 2 to 6 such as 2 or 4 or 6.

15

The functionalized polymers of the present invention are useful as precursors for the preparation of polymer-linker-protein conjugates.

In the context of the present invention the terms alkyl and aryl have the following meaning:

20

The group "alkyl" is a linear, branched or cyclic substituted or unsubstituted C1 to C20, preferably C1 to C9, more preferably C1 to C4 alkyl group. The alkyl group may be unsubstituted or substituted with aryl groups, halogen, nitro, ether, alkoxy, amino or carboxylic groups. Preferably, the alkyl groups are unsubstituted. Further, one or more non adjacent carbon atoms of the alkyl group may be replaced by hetero atoms selected from O, S and N. The hetero atoms are optionally substituted with hydrogen, alkyl as mentioned above or aryl in accordance with their valency. Preferred alkyl groups are methyl, ethyl, iso-propyl, n-propyl, iso-butyl, n-butyl, sec-butyl and tert-butyl.

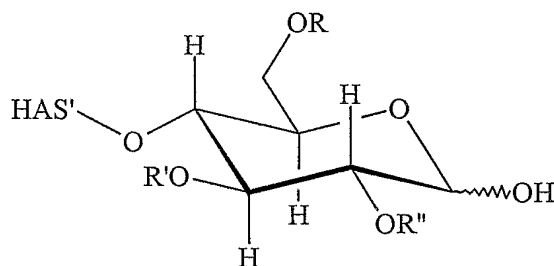
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30 The group "aryl" is preferably a C6-aryl group. The aryl group may be unsubstituted or substituted with linear, branched or cyclic alkyl groups as mentioned above. The aryl group may further contain one or more hetero atoms in the ring, preferably selected from N, O and S. Most preferably the alkyl group is phenyl or toluyl.

"polymer"

Suitable "polymer"s are polysaccharides, preferably selected from the group consisting of starch, cellulose, dextran, gum arabic, xanthan gum, inulin, ghatti gum, pectin, guar gum, gum tragacanth, agar, algin, karaya gum, carrageenan, scleroglucan, fucellaran, arabinogalacton and locust bean gum. In the context of the present invention "polysaccharides" means polysaccharides as mentioned above as well as derivatives thereof. Derivatives of the polysaccharides mentioned above and the preparation thereof as well as the polysaccharides mentioned above are known by a person skilled in the art. Preferred polysaccharides are selected from the group consisting of starch, preferably hydroxyalkyl starch (HAS), and dextran.

In the context of the present invention, the term "hydroxyalkyl starch" (HAS) refers to a starch derivative which has been substituted by at least one hydroxyalkyl group. A preferred hydroxyalkyl starch of the present invention has a constitution according to formula (VI)



VI

wherein the reducing end of the starch molecule is shown in the non-oxidized form and the terminal saccharide unit is shown in the hemiacetal form which, depending on e.g. the solvent, may be in equilibrium with the aldehyde form. The abbreviation HAS' as used in the context of the present invention refers to the HAS molecule without the terminal saccharide unit at the reducing end of the HAS molecule.

The term hydroxyalkyl starch as used in the present invention is not limited to compounds where the terminal carbohydrate moiety comprises hydroxyalkyl groups R, R', and/or R'' as depicted, for the sake of brevity, in formula (VI), but also refers to compounds in which at least one hydroxyalkyl group is present anywhere, either in the terminal carbohydrate moiety and/or in the remaining part of the starch molecule, HAS', is substituted by a hydroxyalkyl group R, R', or R''.

Hydroxyalkyl starch comprising two or more different hydroxyalkyl groups are also possible.

5 The at least one hydroxyalkyl group comprised in HAS may contain two or more hydroxy groups. According to a preferred embodiment, the at least one hydroxyalkyl group comprised HAS contains one hydroxy group.

10 The expression "hydroxyalkyl starch" also includes derivatives wherein the alkyl group is mono- or polysubstituted. In this context, it is preferred that the alkyl group is substituted with a halogen, especially fluorine, or with an aryl group. Furthermore, the hydroxy group of a hydroxyalkyl group may be esterified or etherified.

15 Furthermore, instead of alkyl, also linear or branched substituted or unsubstituted alkene groups may be used.

Hydroxyalkyl starch is an ether derivative of starch. Besides of said ether derivatives, also other starch derivatives can be used in the context of the present invention. For example, derivatives are useful which comprise esterified hydroxy groups. These derivatives may be e.g. derivatives of unsubstituted mono- or dicarboxylic acids with 2-12 carbon atoms or of substituted derivatives thereof. Especially useful are derivatives of unsubstituted monocarboxylic acids with 2-6 carbon atoms, especially derivatives of acetic acid. In this context, acetyl starch, butyl starch and propyl starch are preferred.

25 Furthermore, derivatives of unsubstituted dicarboxylic acids with 2-6 carbon atoms are preferred.

In the case of derivatives of dicarboxylic acids, it is useful that the second carboxy group of the dicarboxylic acid is also esterified. Furthermore, derivatives of monoalkyl esters of dicarboxylic acids are also suitable in the context of the present invention.

30 For the substituted mono- or dicarboxylic acids, the substitute groups may be preferably the same as mentioned above for substituted alkyl residues.

Techniques for the esterification of starch are known in the art (see e.g. Klemm D. et al, Comprehensive Cellulose Chemistry Vol. 2, 1998, Wiley-VCH, Weinheim, New York, especially chapter 4.4, Esterification of Cellulose (ISBN 3-527-29489-9).

- 5 According to a preferred embodiment of the present invention, hydroxyalkyl starch according to above-mentioned formula (VI) is employed. In formula (VI), the saccharide ring described explicitly and the residue denoted as HAS' together represent the preferred hydroxyalkyl starch molecule. The other saccharide ring structures comprised in HAS' may be the same as or different from the explicitly described saccharide ring.

10

As far as the residues R, R' and R'' according to formula (VI) are concerned there are no specific limitations. According to a preferred embodiment, R, R' and R'' are independently hydrogen or a hydroxyalkyl group, a hydroxyaryl group, a hydroxyaralkyl group or a hydroxyalkaryl group having of from 2 to 10 carbon atoms in the respective alkyl residue.

- 15 Hydrogen and hydroxyalkyl groups having of from 2 to 10 are preferred. More preferably, the hydroxyalkyl group has from 2 to 6 carbon atoms, more preferably from 2 to 4 carbon atoms, and even more preferably 2 carbon atoms. "Hydroxyalkyl starch" therefore preferably comprises hydroxyethyl starch, hydroxypropyl starch and hydroxybutyl starch, wherein hydroxyethyl starch and hydroxypropyl starch are particularly preferred and hydroxyethyl
20 starch is most preferred.

The alkyl, aryl, aralkyl and/or alkaryl group may be linear or branched and suitably substituted.

- 25 Thus, R, R' and R'' preferably may be hydroxyhexyl, hydroxypentyl, hydroxybutyl, hydroxypropyl such as 2-hydroxypropyl, 3-hydroxypropyl, 2-hydroxyisopropyl, hydroxyethyl such as 2-hydroxyethyl, hydrogen and the 2-hydroxyethyl group being especially preferred.

- Hydroxyethyl starch (HES) is most preferred for all embodiments of the present invention
30 concerning starch.

Hydroxyethyl starch (HES) is a derivative of naturally occurring amylopectin and is degraded by alpha-amylase in the body. HES is a substituted derivative of the carbohydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95 % by weight. HES

exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in the clinics (Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278; and Weidler et al., 1991, Arzneimittel.-Forschung/Drug Res., 41, 494-498).

5

Amylopectin consists of glucose moieties, wherein in the main chain alpha-1,4-glycosidic bonds are present and at the branching sites alpha-1,6-glycosidic bonds are found. The physical-chemical properties of this molecule are mainly determined by the type of glycosidic bonds. Due to the nicked alpha-1,4-glycosidic bond, helical structures with about six glucose-
10 monomers per turn are produced. The physico-chemical as well as the biochemical properties of the polymer can be modified via substitution. The introduction of a hydroxyethyl group can be achieved via alkaline hydroxyethylation. By adapting the reaction conditions it is possible to exploit the different reactivity of the respective hydroxy group in the unsubstituted glucose monomer with respect to a hydroxyethylation. Owing to this fact, the skilled person is able to
15 influence the substitution pattern to a limited extent.

HES is mainly characterized by the molecular weight distribution and the degree of substitution. There are two possibilities of describing the substitution degree:

1. The degree can be described relatively to the portion of substituted glucose monomers
20 with respect to all glucose moieties.
2. The degree of substitution can be described as the molar substitution, wherein the number of hydroxyethyl groups per glucose moiety are described.

In the context of the present invention, the degree of substitution, denoted as DS, relates to the molar substitution, as described above.

25

HES solutions are present as polydisperse compositions, wherein each molecule differs from the other with respect to the polymerisation degree, the number and pattern of branching sites, and the substitution pattern. HES is therefore a mixture of compounds with different molecular weight. Consequently, a particular HES solution is determined by average
30 molecular weight with the help of statistical means. In this context, M_n is calculated as the arithmetic mean depending on the number of molecules. Alternatively, M_w (or MW), the weight mean, represents a unit which depends on the mass of the HES.

In the context of the present invention, hydroxyethyl starch may preferably have a mean molecular weight (weight mean) of from 1 to 300 kD. Hydroxyethyl starch can further exhibit a preferred molar degree of substitution of from 0.1 to 0.8 and a preferred ratio between C₂ : C₆ substitution in the range of from 2 to 20 with respect to the hydroxyethyl groups.

5

The term "mean molecular weight" as used in the context of the present invention relates to the weight as determined according to Sommermeyer et al., 1987, Krankenhauspharmazie, 8(8), 271-278; and Weidler et al., 1991, Arzneim.-Forschung/Drug Res., 41, 494-498.

10 According to a preferred embodiment of the present invention, the mean molecular weight of hydroxyethyl starch employed is from 1 to 300 kD, more preferably from 2 to 200 kD, more preferably of from 4 to 130 kD, more preferably of from 4 to 70 kD.

15 An example for HES with a mean molecular weight of about 130 kD is Voluven® from Fresenius. Voluven® is an artificial colloid, employed, e.g., for volume replacement used in the therapeutic indication for therapy and prophylaxis of hypovolaemia. The characteristics of Voluven® are a mean molecular weight of 130,000 +/- 20,000 D, a molar substitution of 0.4 and a C₂ : C₆ ratio of about 9:1.

20 Therefore, the present invention also relates to a method and to conjugates as described above wherein the hydroxyalkyl starch is hydroxyethyl starch having a mean molecular weight of from 4 to 70 kD.

25 Preferred ranges of the mean molecular weight are, e.g., 4 to 70 kD or 10 to 70 kD or 12 to 70 kD or 18 to 70 kD or 50 to 70 kD or 4 to 50 kD or 10 to 50 kD or 12 to 50 kD or 18 to 50 kD or 4 to 18 kD or 10 to 18 kD or 12 to 18 kD or 4 to 12 kD or 10 to 12 kD or 4 to 10 kD.

30 According to particularly preferred embodiments of the present invention, the mean molecular weight of hydroxyethyl starch employed is in the range of from more than 4 kD and below 70 kD, such as about 10 kD, or in the range of from 9 to 10 kD or from 10 to 11 kD or from 9 to 11 kD, or about 12 kD, or in the range of from 11 to 12 kD or from 12 to 13 kD or from 11 to 13 kD, or about 18 kD, or in the range of from 17 to 18 kD or from 18 to 19 kD or from 17 to 19 kD, or about 50 kD, or in the range of from 49 to 50 kD or from 50 to 51 kD or from 49 to 51 kD.

As far as the degree of substitution (DS) is concerned, DS is preferably at least 0.1, more preferably at least 0.2, more preferably at least 0.4 and more preferably at least 0.7. Preferred ranges of DS are from 0.1 to 0.8, more preferably from 0.2 to 0.8, more preferably from 0.3 to 0.8 and even more preferably from 0.4 to 0.8, still more preferably from 0.1 to 0.7, more preferably from 0.2 to 0.7, more preferably from 0.3 to 0.7 and more preferably from 0.4 to 0.7. Particularly preferred values of DS are, e.g., 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8, with 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 being more preferred, 0.3, 0.4, 0.5, 0.6, 0.7 or 0.8 being even more preferred, 0.4, 0.5, 0.6, 0.7 or 0.8 being still more preferred and, e.g. 0.4 and 0.7 being particularly preferred.

Particularly preferred combinations of molecular weight of the hydroxyalkyl starch, preferably hydroxyethyl starch, and its degree of substitution DS are, e.g., 10 kD and 0.4 or 10 kD and 0.7 or 12 kD and 0.4 or 12 kD and 0.7 or 18 kD and 0.4 or 18 kD and 0.7 or 50 kD and 0.4 or 50 kD and 0.7.

In a further preferred embodiment the combinations of molecular weight of the hydroxyalkyl starch, preferably hydroxyethyl starch, and its degree of substitution DS are 30 kD and 0.7 or 30 kD and 0.4.

Further preferred combinations of molecular weight of the hydroxyalkyl starch, preferably hydroxyethyl starch, and its degree of substitution DS are 10 kD and 0.8, 12 kD and 0.8, 18 kD and 0.8, 30 kD and 0.8, and 50 kD and 0.8.

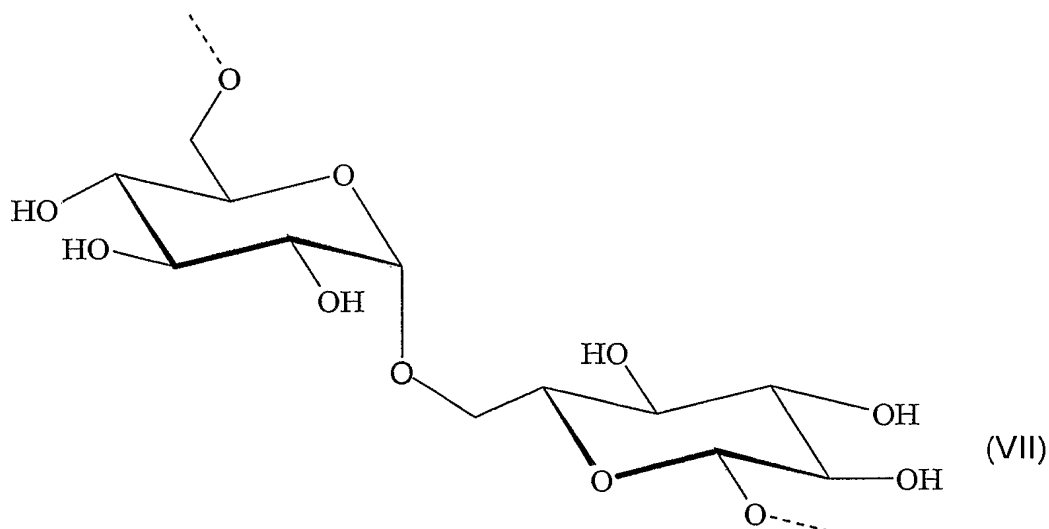
As far as the ratio of C₂ : C₆ substitution is concerned, said substitution is preferably in the range of from 2 to 20, more preferably in the range of from 2 to 15 and even more preferably in the range of from 3 to 12.

According to a further embodiment of the present invention, also mixtures of hydroxyethyl starches may be employed having different mean molecular weights and/or different degrees of substitution and/or different ratios of C₂ : C₆ substitution. Therefore, mixtures of hydroxyethyl starches may be employed having different mean molecular weights and different degrees of substitution and different ratios of C₂ : C₆ substitution, or having different mean molecular weights and different degrees of substitution and the same or about the same

ratio of $C_2 : C_6$ substitution, or having different mean molecular weights and the same or about the same degree of substitution and different ratios of $C_2 : C_6$ substitution, or having the same or about the same mean molecular weight and different degrees of substitution and different ratios of $C_2 : C_6$ substitution, or having different mean molecular weights and the same or about the same degree of substitution and the same or about the same ratio of $C_2 : C_6$ substitution, or having the same or about the same mean molecular weights and different degrees of substitution and the same or about the same ratio of $C_2 : C_6$ substitution, or having the same or about the same mean molecular weight and the same or about the same degree of substitution and different ratios of $C_2 : C_6$ substitution, or having about the same mean molecular weight and about the same degree of substitution and about the same ratio of $C_2 : C_6$ substitution.

In different functionalized polymers and/or conjugates methods according to the present invention, different hydroxyalkyl starches, preferably different hydroxyethyl starches and/or different hydroxyalkyl starch mixtures, preferably different hydroxyethyl starch mixtures, may be employed.

Dextran containing a backbone of D-glucose units linked predominately alpha-D (1,6) with additional 1,3 branching points according to formula (VII), is produced mainly by bacteria or synthetically.

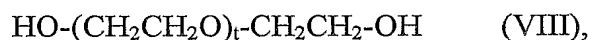


The molecular weight of the dextran is preferably from 4kD to 300kD, more preferably 5kD to 100kD and most preferably 6kD to 40kD. The molecular weights are determined by GPC using suitable commercially available molecular weight standards.

- 5 Further suitable polymers are soluble linear or branched polymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, preferably ethylene glycol homopolymers (PEG) and propylene glycol homopolymers, alkylene glycol copolymers, preferably propylene oxide/ethylene oxide co-polymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride
10 copolymer and polyaminoacids (either homopolymers or random copolymers). In the context of the present invention the polymers as mentioned above as well as derivatives thereof may be employed. The polymers mentioned above and their preparation as well as derivatives thereof are known by a person skilled in the art.
- 15 Preferred polymers are alkylene glycol homopolymers, preferably ethylene glycol homopolymers (PEG) and propylene glycol homopolymers. More preferred are ethylene glycol homopolymers (PEG).

PEGs are polyether diols of the general structure (VIII)

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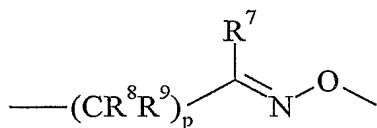
- wherein t is the number of $-(\text{CH}_2\text{CH}_2\text{O})$ -groups. They are commercially available in a variety of molecular weights and low dispersity ($M_w/M_n \leq 1.1$, wherein M_w is the weight average
25 molecular weight and M_n is the number average molecular weight). While the polyether backbone is fairly chemically inert the primary hydroxyl groups are available for derivatization. The molecular weights of PEGs used for the preparation of bioconjugates vary between 1000 and 20000 Da, although in some instances the polymers of higher and lower molecular weights than in this range are utilized. The molecular weights are determined by
30 GPC using suitable commercially available molecular weight standards. Preferably the monoalkyl ether of PEG is employed, wherein the alkyl group is selected from a C1 to C4 alkyl group. More preferably the monomethyl ether of PEG (mPEG) is used. The presence of only one derivatizable terminal group on mPEG or other monoalkyl ethers of PEG minimizes the possibilities for crosslinking and improves the homogeneity of conjugates prepared.

Besides the monoalkyl ether of PEG, preferably mPEG, other functionalized PEG derivatives may be used in the present invention. Suitable examples are halo-substituted derivatives of PEG, sulfonate esters of PEG, amino-PEG, hydrazido-PEG, mercapto-PEG, carboxyl-PEG and its active esters, aldehyde-PEG, cyanuryl chloride-PEG, and epoxide-PEG. The preparation of the derivatives mentioned above as well as further derivatives and their preparation are disclosed in S. Zalipsky, Bioconjugate Chem. 1995, 6, 150-165. Furthermore, some of the PEG derivatives are commercially available.

The functional derivatives of PEG are in general prepared by (i) direct transformation of hydroxyls to the new target functionality and (ii) reaction of the polymer with a bifunctional molecule so that one function forms an attachment to the polymer and the other one remains available for further chemical transformations.

Most preferably the monoalkyl ether of PEG, preferably mPEG, is used in the present invention.

The group X in the functionalized polymers of formula I is a linking group, selected from the group consisting of $-(CR^8R^9)_pO-$, $-(CR^8R^9)_pS-$, $-(CR^8R^9)_pNR^6-$, $-(CR^8R^9)_pOC(O)-$, $-(CR^8R^9)_pC(O)O-$, $-(CR^8R^9)_pC(G)N(R^{10})O-$, $-(CR^8R^9)_pN(R^{11})O-$, and

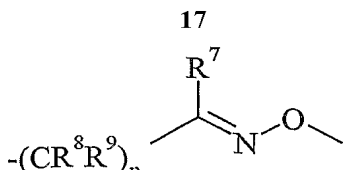


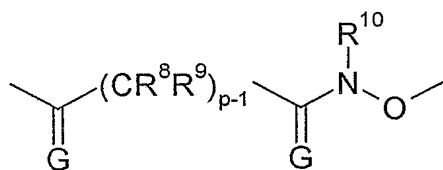
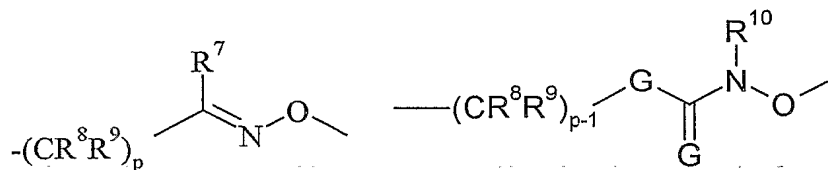
wherein one or more groups $-(CR^8R^9)-$ may be replaced by W, whereby a chemically reasonable group is formed.

W is O, NR^{12} , C(G), preferably O, C(G) and G is S, O, NR^{14} , preferably O.

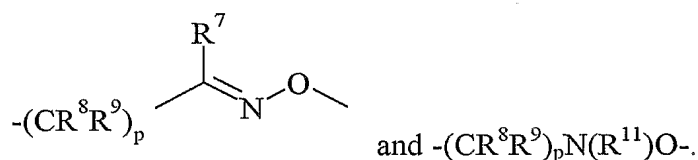
R^6 , R^7 , R^8 , R^9 , R^{10} , R^{11} , R^{12} , R^{14} in the functionalized polymer of formula I are independently hydrogen, alkyl, aryl, preferably hydrogen.

p is 0 to 20, preferably 0 to 10, more preferably 0 to 5, most preferably 0 to 4, even more preferably 0 in the case of polysaccharides and derivatives thereof, and 1 to 4 in the case of polyalkylene glycols and derivatives thereof, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 .

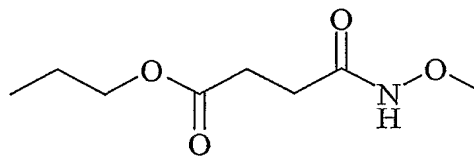
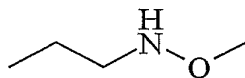
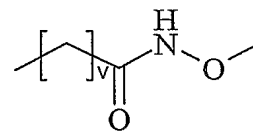
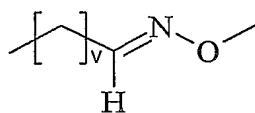
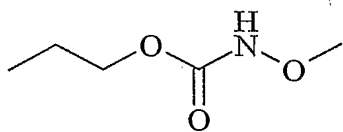
Preferred groups X are $-(\text{CR}^8\text{R}^9)_p$ , $-(\text{CR}^8\text{R}^9)_p\text{C}(\text{G})\text{N}(\text{R}^{10})\text{O}-$, and $-(\text{CR}^8\text{R}^9)_p\text{N}(\text{R}^{11})\text{O}-$, more preferred are



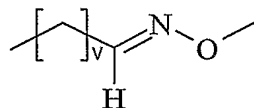
5 $-(\text{CR}^8\text{R}^9)_p\text{N}(\text{R}^{11})\text{O}-$, even more preferred are

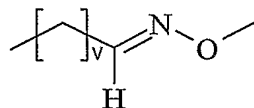


Preferred structures for the linking group X when the polymer is polyalkylene glycol or a derivative thereof are

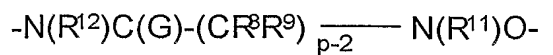
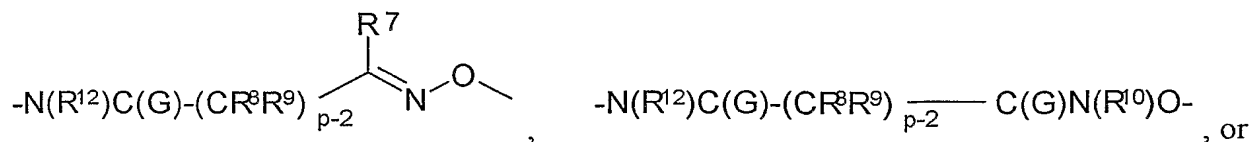


$v = 1$ to 10 , preferably 1 to 4 , more preferably 2 or 3 .



Most preferred is , wherein v is 1 to 10 , preferably 1 to 4 , more preferably 2 to 3 .

With regard to above-mentioned group X, in the case of polyalkylene glycols and derivatives thereof, also the following structures of the linking group X are preferred:



wherein p is 2, 3 or 4, preferably 3 or 4 and most preferably 4, with

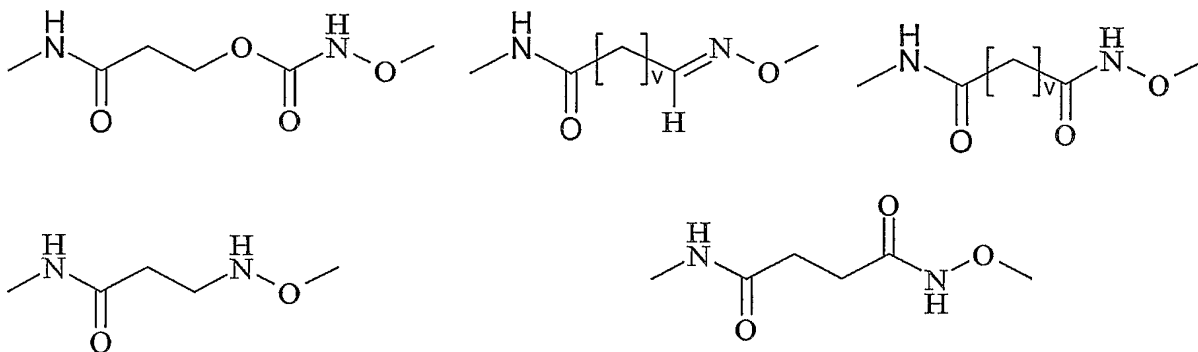
$\text{R}^7, \text{R}^8, \text{R}^9, \text{R}^{10}, \text{R}^{11}, \text{R}^{12}$ hydrogen, alkyl, aryl, preferably hydrogen,

in a further preferred embodiment X is $\text{-N(R}^{12}\text{)C(G)-(CR}^8\text{R}^9\text{)}_{p-3} \text{---G---C(G)N(R}^{10}\text{)O-}$,

wherein p is 5, with

$\text{R}^7, \text{R}^8, \text{R}^9, \text{R}^{10}, \text{R}^{11}, \text{R}^{12}$ hydrogen, alkyl, aryl, preferably hydrogen.

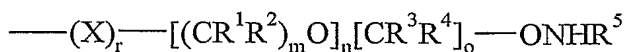
Even more preferred are the following structures:



$v = 1$ to 10 , preferably 1 to 4 , more preferably 2 or 3 .

Most preferred are and , wherein v is 1 to 10 , preferably 1 to 4 , more preferably 2 to 3 .

With regard to above-mentioned group X, preferably in the case of polyalkylene glycols and derivatives thereof, also groups



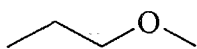
are preferred where $r = 1$ and $X = -(CR^8R^9)_pO-$ or $-(CR^8R^9)_pS-$,

wherein one or more groups $-(CR^8R^9)-$ may be replaced by W, whereby a chemically reasonable group is formed

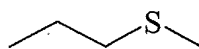
W is O, NR^{12} , C(G), preferably O, C(G) and G is S, O, NR^{14} , preferably O;

- 5 R^8 , R^9 and R^{12} are independently hydrogen, alkyl, aryl, preferably hydrogen; and
p is 2, 3 or 4.

In these cases, the following structures



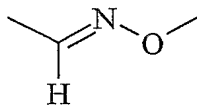
and



and $-N(R^{12})C(G)-(CR^8R^9)_{p-2}-S-$

- 10 of the linking group X are especially preferred, wherein G, R^8 , R^9 , R^{12} and p is 3 or 4, preferably 4.

When the polymer is a polysaccharide, preferably selected from the group consisting of dextran or a derivative thereof or starch or a derivative thereof as mentioned before, X is most
15 preferably



or $-N(H)O-$.

- In the group $-[(CR^1R^2)_mO]_n[CR^3R^4]_o-$ in formula I R^1 , R^2 , R^3 , and R^4 are independently hydrogen, alkyl or aryl as defined above, preferably methyl or hydrogen, more preferably
20 hydrogen. m is 2 to 4, preferably 2, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2 . Preferably $-(CR^1R^2)_m-$ is $-CH_2CH_2-$ or $-CH(CH_3)CH_2-$ or $-CH_2CH(CH_3)-$, more preferably $-(CR^1R^2)_m-$ is $-CH_2CH_2-$.

- n is 0 to 20, preferably 0 to 10, more preferably 1 to 5, most preferably 1 or 2 and even more
25 preferably 1.

o is 0 to 20, preferably 0 to 10, more preferably 0 or 2, wherein in the case of $n = 0$, o is not 0, in a preferred embodiment o is 2 to 20, preferably 2 to 10, more preferably 2, wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4 .

30

In a most preferred embodiment of the present invention $-[(CR^1R^2)_mO]_n[CR^3R^4]_o-$ is $-CH_2CH_2OCH_2CH_2-$.

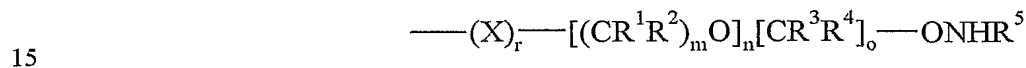
In a further preferred embodiment of the present invention $-\text{[(CR}^1\text{R}^2)_m\text{O]}_n\text{[CR}^3\text{R}^4)_o-$ is $-\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2-$.

5 R^5 in formula I is hydrogen, alkyl, aryl as mentioned before, preferably hydrogen.

The group $-\text{[(CR}^1\text{R}^2)_m\text{O]}_n\text{[CR}^3\text{R}^4)_o-\text{ONHR}^5$ in formula I is therefore in a most preferred embodiment of the present invention $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2-\text{ONH}_2$.

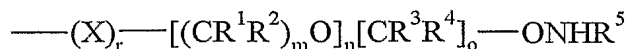
10 The group $-\text{[(CR}^1\text{R}^2)_m\text{O]}_n\text{[CR}^3\text{R}^4)_o-\text{ONHR}^5$ in formula I is therefore in a further most preferred embodiment of the present invention $-\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2-\text{ONH}_2$.

The group



is covalently linked with least one terminal group or least one centrally located group of the "polymer".

20 In general any suitable group of the polymer may be used for the covalent linkage, depending on the polymer employed. Most preferably the group



is linked with the polymer employed, preferably selected from the group consisting of starch, dextran and polyalkylene glycol or derivatives thereof, more preferably hydroxyalkyl starch, whereby hydroxyethyl starch (HES) is preferred, dextran and the monomethyl ether of polyethylene glycol (mPEG), by an oxygen comprising group of the polymer. The oxygen group may be a carbonyl group, preferably a keto group, a hemiacetal group or an aldehyde group, more preferably a hemiacetal group or an aldehyde group. In the case of polyalkylene glycols, the oxygen comprising group may also be a group OR'''' , e.g. a carboxylic acid ester or a carbonate, which is derived from the reaction of the polyalkylene glycol or a derivative thereof with alcohols, whereby preferred alcohols are selected from the group consisting of N-hydroxy succinimides such as N-hydroxy succinimide or Sulfo-N-hydroxy succinimide, suitably substituted phenols such as p-nitrophenol, o,p-dinitrophenol, o,o'-dinitrophenol, trichlorophenol such as 2,4,6-trichlorophenol or 2,4,5-trichlorophenol,

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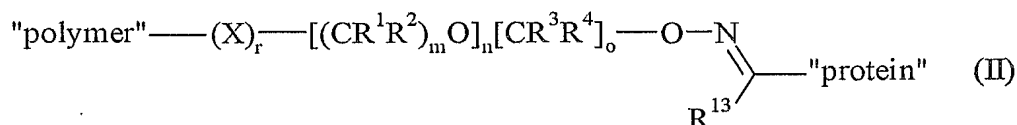
trifluorophenol such as 2,4,6-trifluorophenol or 2,4,5-trifluorophenol, pentachlorophenol, pentafluorophenol, or hydroxyazoles such as hydroxy benzotriazole. Especially preferred are N-hydroxy succinimides, with N-hydroxy succinimide and Sulfo-N-hydroxy succinimide being especially preferred. However, it is also possible that the linkage with the group X is achieved by other groups than the oxygen comprising groups mentioned before. The linkage of



with the polymer will be explained in detail later.

- 10 The functionalized polymer of formula I is suitable for the preparation of stable conjugates with proteins.

In a further embodiment the present invention therefore relates to a conjugate of formula II

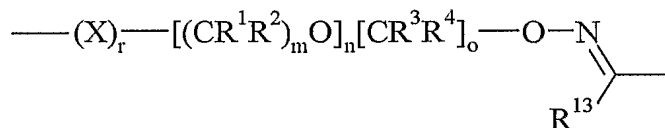


15 wherein the symbols "polymer", R^1 , R^2 , R^3 , R^4 , m , n , o , r , X , and p have the same meaning as mentioned before and

R^{13} is hydrogen, alkyl, aryl, preferably hydrogen or methyl, and

"protein" is an amino acid sequence prepared by reaction of at least 2 amino acids

20 wherein in the conjugate of formula II the group



is covalently linked with least one terminal group or least one centrally located group of the "polymer" and the "protein", preferably with an oxidized N-terminal amino acid or an oxidized carbohydrate side chain of the "protein".

"protein"

The term "protein" as used in the context of the present invention, relates to any amino acid sequence having at least 2, preferably at least 5, more preferably at least 10, more preferably

at least 15, more preferably at least 20, more preferably at least 25, more preferably at least 30, more preferably at least 35, more preferably at least 40, more preferably at least 45 and still more preferably at least 50 amino acids.

- 5 The protein can be produced by chemical synthetic procedures or can be of any human or another mammalian source and can be obtained by purification from naturally occurring sources.

According to the present invention, the protein can be a growth factor, a cytokine, an
10 activator, an inhibitor, an enzyme, an antibody, an antigen, a transport protein, a bioadhesion protein, a hormone, a receptor, a suppressor, or a functional derivative or a fragment thereof. The term "functional derivative or fragment" as used in the context of the present invention relates to a derivative or fragment that maintains the desired biological property or activity of
15 the original molecule totally or partially, e.g. at least 10 %, more preferably at least 20 %, more preferably at least 30 %, more preferably at least 40 %, more preferably at least 50 %, more preferably at least 60 %, more preferably at least 70 %, more preferably at least 80 % and especially preferably at least 90 % of the desired biological property or activity of the original molecule. Particularly preferred examples of such fragments are, e.g., antibody
fragments.

20

Examples of proteins are erythropoietin (EPO) such as recombinant human EPO (rhEPO), colony-stimulating factors (CSF), such as G-CSF like recombinant human G-CSF (rhG-CSF), alpha-Interferon (IFN alpha), beta-Interferon (IFN beta) or gamma-Interferon (IFN gamma), such as IFN alpha and IFN beta like recombinant human IFN alpha or IFN beta (rhIFN alpha
25 or rhIFN beta), interleukines, e.g. IL-1 to IL-18 such as IL-2 or IL-3 like recombinant human IL-2 or IL-3 (rhIL-2 or rhIL-3), serum proteins such as coagulation factors II-XIII like factor VIII, factor VII, factor IX, alpha1-antitrypsin (A1AT), activated protein C (APC), plasminogen activators such as tissue-type plasminogen activator (tPA), such as human tissue plasminogen activator (hTPA), AT III such as recombinant human AT III (rhAT III),
30 myoglobin, albumin such as bovine serum albumin (BSA), growth factors, such as epidermal growth factor (EGF), thrombocyte growth factor (PDGF), fibroblast growth factor (FGF), brain-derived growth factor (BDGF), nerve growth factor (NGF), B-cell growth factor (BCGF), brain-derived neurotrophic growth factor (BDNF), ciliary neurotrophic factor (CNTF), transforming growth factors such as TGF alpha or TGF beta, BMP (bone

morphogenic proteins), growth hormones such as human growth hormone, tumor necrosis factors such as TNF alpha or TNF beta, somatostatine, somatotropine, somatomedines, hemoglobin, hormones or prohormones such as insulin, gonadotropin, melanocyte-stimulating hormone (alpha-MSH), triptorelin, hypthalamic hormones such as antidiuretic hormones (ADH and oxytocin as well as releasing hormones and release-inhibiting hormones, parathyroid hormone, thyroid hormones such as thyroxine, thyrotropin, thyroliberin, prolactin, calcitonin, glucagon, glucagon-like peptides (GLP-1, GLP-2 etc.), exendines such as exendin-4, leptin, vasopressin, gastrin, secretin, integrins, glycoprotein hormones (e.g. LH, FSH etc.), melanocyte-stimulating hormones, lipoproteins and apo-lipoproteins such as apo-B, apo-E, apo-L_a, immunoglobulins such as IgG, IgE, IgM, IgA, IgD and fragments thereof, hirudin, tissue-pathway inhibitor, plant proteins such as lectin or ricin, bee-venom, snake-venom, immunotoxins, antigen E, alpha-proteinase inhibitor, ragweed allergen, melanin, oligolysine proteins, RGD proteins or optionally corresponding receptors for one of these proteins; or a functional derivative or fragment of any of these proteins or receptors.

Preferred enzymes are, e.g., carbohydrate-specific enzymes, proteolytic enzymes, oxidases, oxidoreductases, transferases, hydrolases, lyases, isomerases, kinases and ligases. Specific non-limiting examples are asparaginase, arginase, arginin deaminase, adenosin deaminase, glutaminase, glutaminase-asparaginase, phenylalanin, tryptophanase, tyrosinase, superoxide dismutase (SOD), endotoxinase, catalase, peroxidase, kallikrein, trypsin, chymotrypsin, elastase, thermolysin, lipase, uricase, adenosine diphosphatase, purine nucleoside phosphorylase, bilirubin oxidase, glucose oxidase, glucodase, gluconate oxidase, galactosidase, glucocerebrosidase, glucuronidase, hyaluronidase, tissue factor, streptokinase, urokinase, MAP-kinases, DNAses, RNAses, lactoferrin and functional derivatives or fragments thereof.

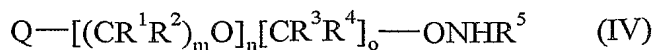
According to preferred embodiments of the present invention, the protein is selected from the group consisting of EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII, APC.

With the conjugates of the present invention it is intended to improve the circulatory life time *in vivo* of the proteins employed and to reduce the antigenicity and immunogenicity of the proteins compared to the proteins before conjunction.

In a further embodiment the present invention relates to a process for preparing a functionalized polymer comprising the step of reacting a polymer of formula III

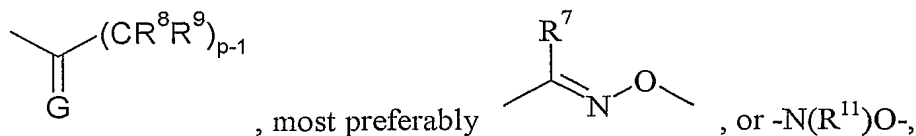
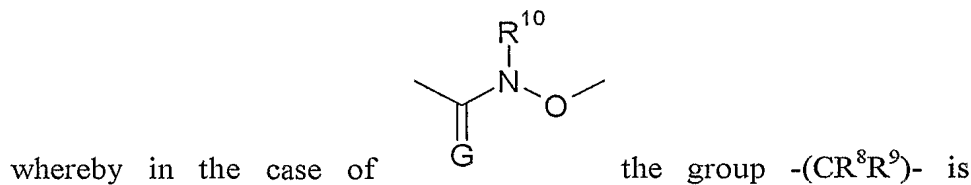
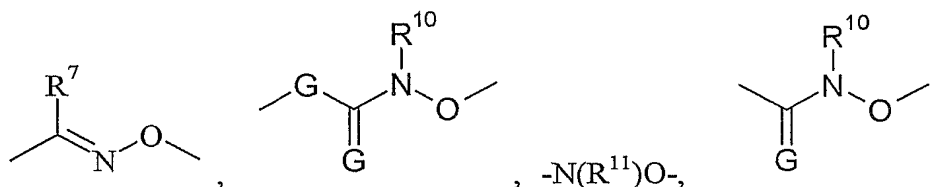
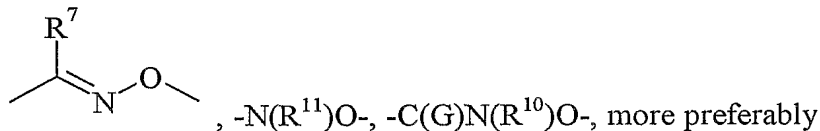
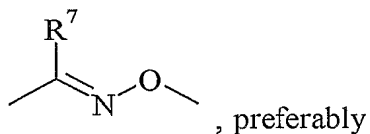


5 with a compound of formula IV



wherein the symbols "polymer", R^1 , R^2 , R^3 , R^4 , R^5 , m , n , o have the same meaning as mentioned before and

10 Y and Q are functional groups, which are suitable to react together to give one of the following linking groups $-\text{O}-$, $-\text{S}-$, $-\text{NR}^6-$, $-\text{OC}(\text{O})-$, $-\text{C}(\text{O})\text{O}-$, $-\text{C}(\text{G})\text{N}(\text{R}^{10})\text{O}-$, $-\text{N}(\text{R}^{11})\text{O}-$,



20 in a further preferred embodiment Y and Q are functional groups, which are suitable to react together to give one of the following linking groups $-\text{O}-$ or $-\text{S}-$;

wherein in the polymer of formula III one or more groups $-(CR^8R^9)-$ may be replaced by W, whereby a chemically reasonable group is formed;

W is O, NR^{12} , C(G), preferably O, C(G);

G is S, O, NR^{14} , preferably O;

5 $R^6, R^7, R^8,$

$R^9, R^{10},$

R^{11}, R^{12}, R^{14} independently hydrogen, alkyl, aryl, preferably hydrogen,

p 0 to 20, preferably 0 to 10, more preferably 0 to 5, most preferably 0 to 4, even more preferably 0 in the case of polysaccharides and derivatives thereof, and 1
10 to 4 in the case of polyalkylene glycols and derivatives thereof, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 ;

wherein the group



is covalently linked with terminal groups or centrally located groups of the "polymer".

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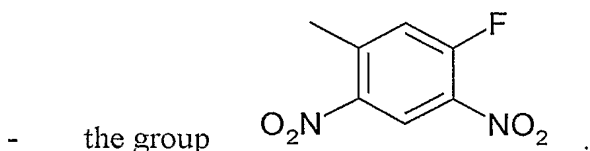
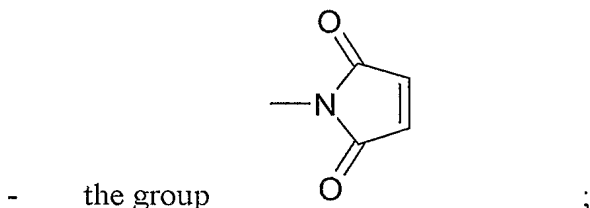
Suitable groups Y are the following functional groups, among others:

- C-C-double bonds or C-C-triple bonds or aromatic C-C-bonds;
- the thio group or the hydroxy groups;
- alkyl sulfonic acid hydrazide, aryl sulfonic acid hydrazide;
- 20 - 1,2-dioles;
- 1,2 amino-thioalcohols;
- azides;
- 1,2-aminoalcohols;
- the amino group $-NH_2$ or derivatives of the amino groups comprising the structure unit -
25 NH- such as aminoalkyl groups, aminoaryl group, aminoaralkyl groups, or alkarlyaminogroups;
- the hydroxylamino group $-O-NH_2$, or derivatives of the hydroxylamino group comprising the structure unit $-O-NH-$, such as hydroxylalkylamino groups, hydroxylaryl amino groups, hydroxylaralkylamino groups, or hydroxalalkaryl amino
30 groups;
- alkoxyamino groups, aryloxyamino groups, aralkyloxyamino groups, or alkaryloxyamino groups, each comprising the structure unit $-NH-O-$;
- residues having a carbonyl group, $-Q'-C(=G)-M$, wherein G is O or S, and M is, for example,

- -OH or -SH;
- an alkoxy group, an aryloxy group, an aralkyloxy group, or an alkaryloxy group;
- an alkylthio group, an arylthio group, an aralkylthio group, or an alkarylthio group;
- 5 -- an alkylcarbonyloxy group, an arylcarbonyloxy group, an aralkylcarbonyloxy group, an alkarylcarbonyloxy group;
- activated esters such as esters of hydroxylamines having imid structure such as N-hydroxysuccinimide or having a structure unit O-N where N is part of a heteroaryl compound or, with G = O and Q' absent, such as aryloxy compounds with a
- 10 substituted aryl residue such as pentafluorophenyl, paranitrophenyl or trichlorophenyl;

wherein Q' is absent or NH or a heteroatom such as S or O;

- -NH-NH₂, or -NH-NH-;
- -NO₂;
- 15 - the nitril group;
- carbonyl groups such as the aldehyde group or the keto group, or a group, which can be converted into an aldehyde group or a keto group, e.g. an acetal or hemiacetal group or a ketal or hemiketal group;
- the carboxy group or an ester group;
- 20 - the -N=C=O group or the -N=C=S group;
- vinyl halide groups such as the vinyl iodide or the vinyl bromide group or triflate;
- -C≡C-H;
- -(C=NH₂Cl)-OAlkyl
- groups -(C=O)-CH₂-Hal wherein Hal is Cl, Br, or I;
- 25 - -CH=CH-SO₂-;
- a disulfide group comprising the structure -S-S-;



A further suitable group Y is the maleimide group.

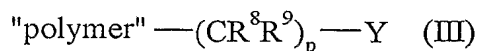
Preferred groups Y are functional groups are groups comprising a carbonyl group, preferably groups comprising an aldehyde group, a keto group, a carboxy group, an ester group, a halogen group, preferably Br or I or OTf ($=O-SO_2CF_3$), or a group, which can be converted into an aldehyde group or a keto group, e.g. an acetal or hemiacetal group or a ketal or hemiketal group.

Further preferred groups Y are residues having a carbonyl group, $-C(=G)-M$, wherein G is O, and M is an activated ester such as an ester of a hydroxylamine having imid structure such as N-hydroxysuccinimide.

In the case that the polymer is a polysaccharide, preferably selected from the group consisting of starch and derivatives thereof and dextran and derivatives thereof, Y is preferably an aldehyde group or an acetal or hemiacetal group. In this case p is preferably 0.

In the case that the polymer is a polyalkylene glycol such as a polyethylene glycol, Y is preferably an aldehyde group or an ester group, preferably a reactive ester group, such as an ester of a hydroxylamine having imid structure such as N-hydroxysuccinimide, a hydroxy group ($-OH$) or a thio group ($-SH$). More preferably, in case Y is a hydroxy group or a thio group, Q is a halogen group, preferably Br or I, or the OTf group. According to this embodiment of the present invention where $X = -(CR^8R^9)_pO-$ or $-(CR^8R^9)_pS-$, the compound according to formula (IV) to be reacted with the polymer according to formula (III) may be employed with the functional group $-ONHR^5$ in a protected form which is de-protected after reaction of (III) with (IV). As protecting group, among others, the phthalimide protecting group may be mentioned which can be removed by reacting the reaction product of (III) and (IV) with hydrazine to give the terminal functional group $-ONHR^5$. However, each suitable protecting which, after de-protection, results in $-ONHR^5$, may be employed as well.

The polymers of formula III



are prepared by methods known by a person skilled in the art.

If polyalkylene glycols, preferably polyethylene glycol (PEG) or the monomethyl ether of PEG (mPEG), are employed, suitable methods for preparing the polymers of formula III are mentioned in S. Zalipsky, Bioconjugate Chem. 1995, 6, 150-165.

- 5 If polysaccharides, preferably starch or dextran or derivatives thereof, are employed, the polymers of formula III are polysaccharides, the group Y is preferably an aldehyde group or a hemiacetal group or an equilibrium of both.

10 Preferably, the aldehyde group Y in the polysaccharide is its reducing end, being in an equilibrium between an aldehyde and a hemiacetal form. If the polymer is a polysaccharide, p in the polymer of formula III is most preferably 0. The linkage between the compound of formula IV and the polymer of formula III, wherein the polymer is a polysaccharide or a derivative thereof is shown in scheme 2.

- 15 The linkage between the compound of formula IV and the polymer of formula III, wherein the polymer is a polyalkylene glycol or a derivative thereof is shown in scheme 1.

To prepare the polysaccharide ("polymer"-(CR⁸R⁹)_p-Y of formula III) for use in the reaction with the compound of formula IV the polysaccharide may be oxidized so as to create thereon
20 a substantial number of aldehyde groups. This can be accomplished by a variety of oxidation processes, the preferred one being reaction with a periodate (sodium oder potassium). This reaction can take place in aqueous solution at low temperature, e.g. 0 to 5 °C, using an appropriate quantity of sodium periodate, chosen according to the desired degree of oxidation.

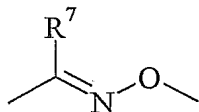
- 25 The reaction is complete in about 10 min to 4 hours. Ultrafiltration or dialysis can be used to remove undesirable low molecular weight salts and polysaccharide components, thereby offering a means of controlling the molecular weight range of oxidized polysaccharide to be reacted with the compound of formula IV. The oxidized polysaccharide can be used directly or is suitably recovered, e.g. by lyophilization, and redissolved for the reaction with the
30 compound of formula IV.

However, it is preferred to employ the polysaccharide or a derivative thereof, whereby preferred polysaccharides are mentioned before, without treatment of the polysaccharide by oxidation. The inventors found that no prior treatment of the polysaccharide is necessary,

when a compound of formula IV, especially a preferred compound of formula IV as mentioned before, is employed.

Suitable groups Q are also functional groups as mentioned above, whereby the functional groups Y and Q are chosen in a way that one of the following group is obtained.

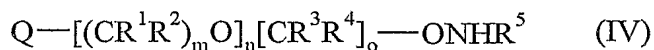
-O-, -S-, -NR⁶-, -OC(O)-, -C(O)O-, -C(G)N(R¹⁰)O-, -N(R¹¹)O-,



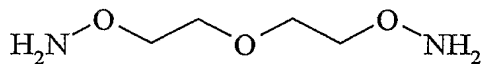
Q is preferably H₂N-O-.

The group Y of the polymer of formula III is therefore preferably a group which is reactable with the group -O-NH₂. Preferred groups Y are therefore aldehyde groups, keto groups, carboxy groups, carbonate groups, and activated carboxy groups, for example ester groups, lactone groups, and amide groups. Further suitable groups are halide and pseudo halide groups and the like, for example Cl, Br, I, and OTf. Most preferably, Y is an aldehyde group (being in the case of polysaccharides - in equilibrium with a hemiacetal form). In a further preferred embodiment Y is an activated ester group such as an ester of a hydroxylamine having imid structure such as N-hydroxysuccinimide.

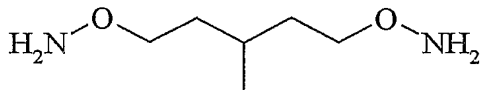
If Q is H₂N-O- the compounds of formula IV



are most preferably

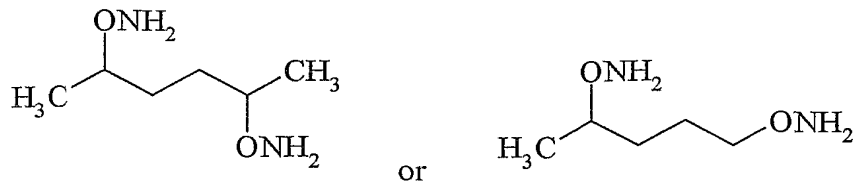


According to another embodiment of the present invention, a compound according to formula (IV) having the structure



is also preferred if Q is H₂N-O-.

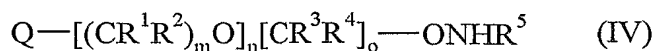
As examples of compounds having two -O-NH₂ groups, also compounds such as



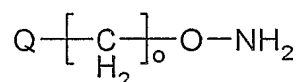
may be mentioned.

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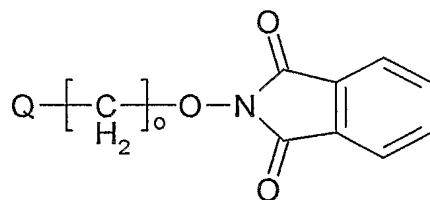
If Q is a halogen group, preferably Br or I, or the OTf group, most preferably Br or I, the compounds of formula IV



10 are most preferably



with o = 2 to 10, more preferably 2 to 8 and especially preferably 2 to 6 such as 2, 4 or 6, and where -O-NH₂ may be present in its protected form such as protected with the phthalimide protecting group such that, for example, the compound according to formula (IV) may be

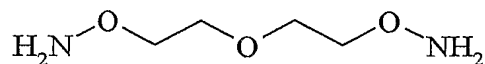


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The preparation of compounds of formula (IV) of a structure as shown above, wherein o is 2 - 6 and Q is Br is described in Bauer et al., J. Org. Chem. 1963, 28, p. 1604. Other compounds of formula (IV) having a structure as shown above are for example obtained by an analogous method.

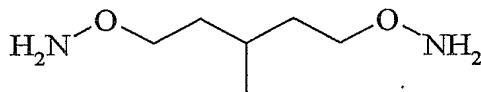
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In a further embodiment the present invention therefore relates to a process as mentioned above, wherein the compound of formula IV is



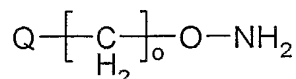
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In yet a further embodiment the present invention therefore relates to a process as mentioned above, wherein the compound of formula IV is



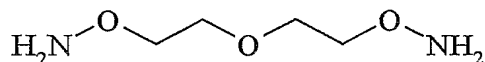
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In yet a further embodiment the present invention therefore relates to a process as mentioned above, wherein the compound of formula IV is



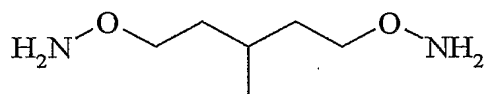
with $o = 2$ to 10 , more preferably 2 to 8 and especially preferably 2 to 6 such as 2 , 4 or 6 , and
10 where $-\text{O}-\text{NH}_2$ may be present in its protected form.

The compounds of formula IV are prepared by methods known by a person skilled in the art. The preparation of



15 is for example disclosed in D. Boturyn et al. Tetrahedron 53 (1997) 5485-5492. Other compounds of formula IV are for example obtained by an analogous method.

For example, as to the synthesis of the compound

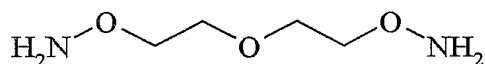


20 reference is made to the respective example hereinunder.

In a preferred embodiment of the process of the present invention for the preparation of functionalized polymers, the polymer of formula III is dissolved in an organic solvent, for example dichloromethane, dimethylformamide, or dimethylacetamide, or in an aqueous
25 system, for example in a sodium acetate buffered aqueous system of pH 4.5 to 9, preferably 5 to 8. The compound of formula IV is added to the solution of the polymer. The addition is usually carried out at a temperature of from 0 to 80°C , preferably 0 to 60°C , more preferably 20 to 40°C . The mixture is agitated by stirring or shaking at said temperature usually for 1 to 48 h, preferably 2 to 24 h, more preferably 2 to 16 h, when the reaction is carried out in an

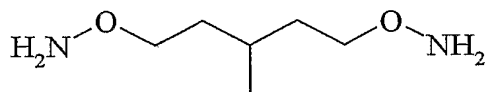
organic solvent and usually for 1 to 48 h, preferably 2 to 24 h, more preferably 2 to 16 h, when the reaction is carried out in an aqueous system. The product is precipitated by adding a solvent or a solvent mixture, wherein the product is insoluble or has a low solubility. Suitable solvents for precipitation of the product depend on the nature of the product. In one embodiment the product is precipitated by adding an alcohol, preferably 2-propanol or ethanol, and incubation at a temperature usually of from -60 to 20 °C, preferably -20 to 20 °C. In a further embodiment the product is precipitated by a mixture of an alcohol with a low boiling polar organic solvent, for example acetone. A suitable solvent mixture is ethanol and acetone, for example a 1 : 1 mixture of ethanol and acetone, indicating equal volumes of said solvents, and incubation at a temperature usually of from -60 to 20 °C, preferably -20 to 20 °C. The precipitated product is collected, for example by centrifugation at low temperatures of in general from 0 to 20 °C, preferably 0 °C, re-suspended, preferably with the solvent or solvent mixture, in one embodiment with the alcohol, which was used for precipitation, at temperatures usually of from -60 to 20 °C, preferably -20 to 20 °C, and incubated usually at the same temperature for in general 0.5 to 20 h, preferably 1 to 3 h. The obtained product is usually worked up further by centrifugation, dissolving of the product in water, dialysing in water for usually 12 to 72 h, preferably 15 to 48 h, more preferably 15 to 25 h and lyophilizing.

In the following schemes, examples for the process for preparing functionalized polymers by reaction of a polymer of formula III and a compound of formula IV, preferably



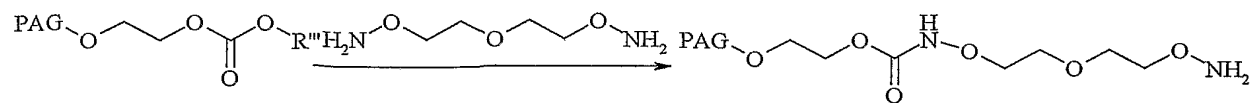
are given (schemes 1a and 2).

In scheme 1b examples for the process for preparing functionalized polymers by reaction of a polymer of formula III and a compound of formula IV, preferably

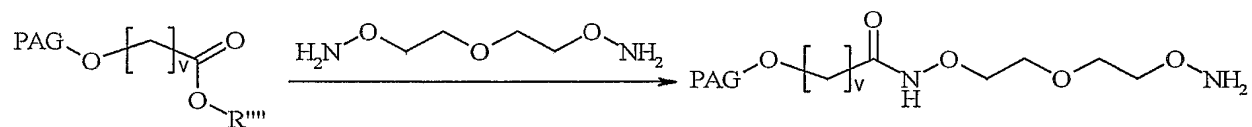
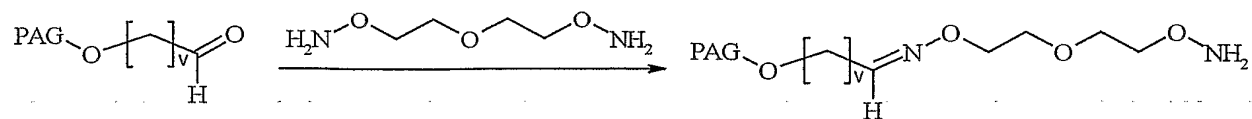


are given.

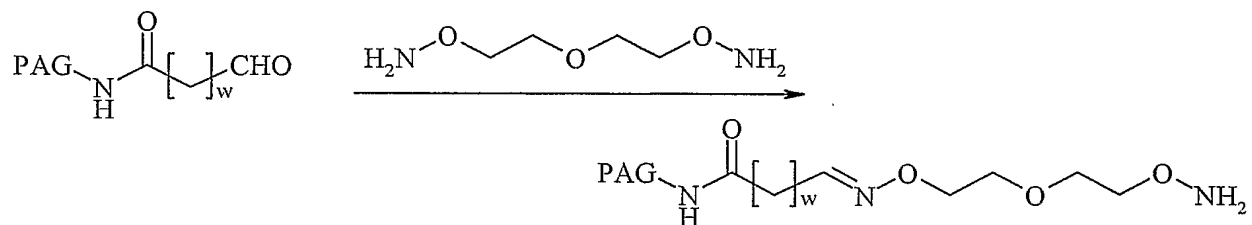
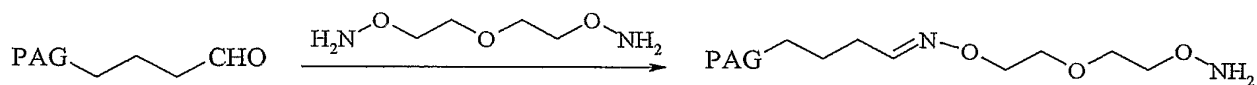
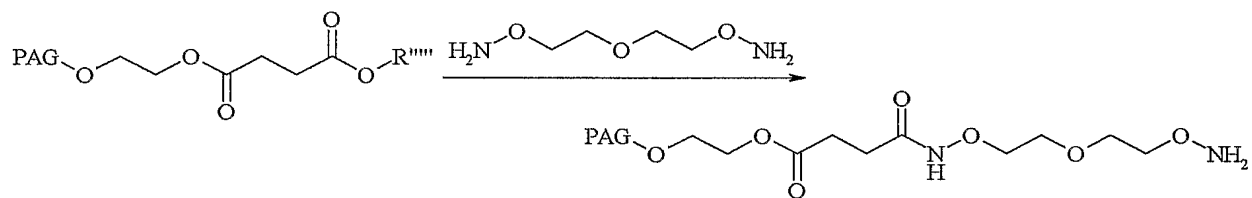
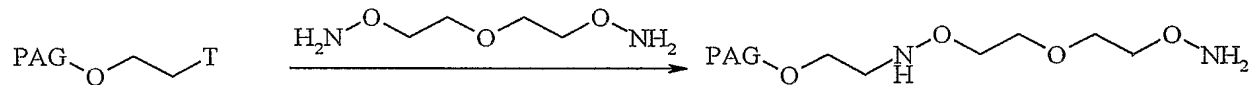
Scheme 1a: polyalkylene glycol (PAG)-derivatives



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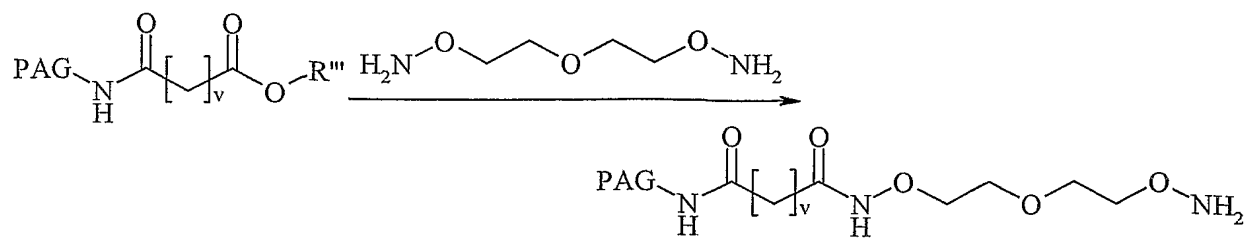


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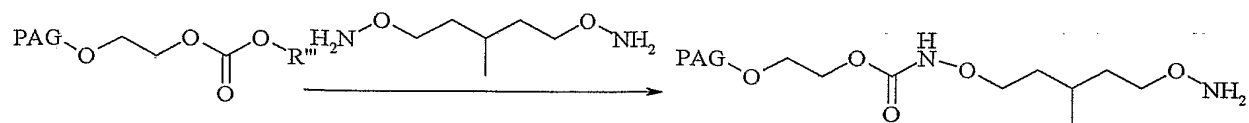


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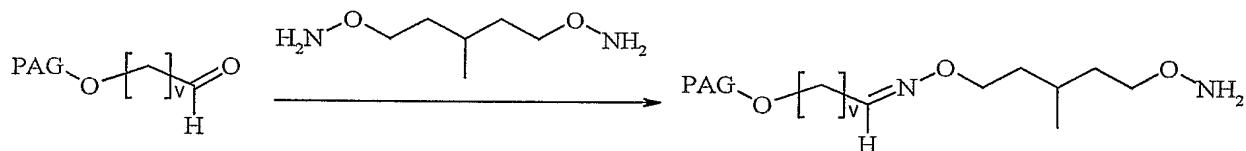
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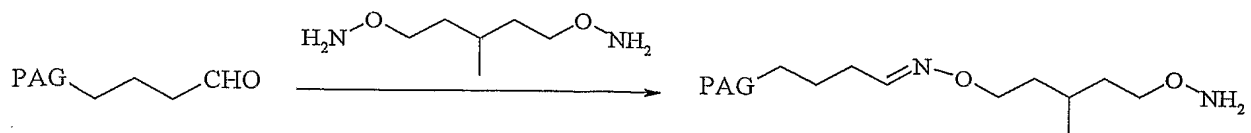
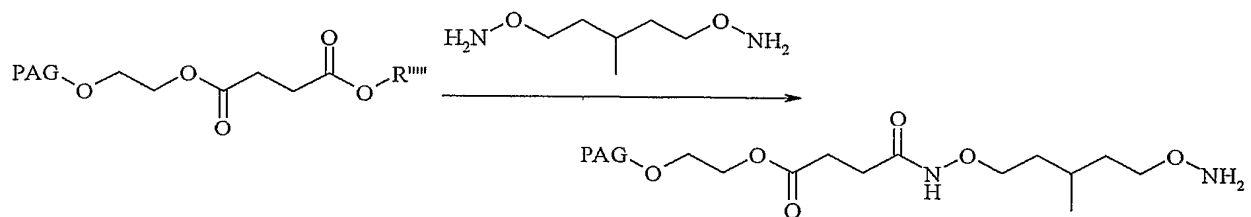
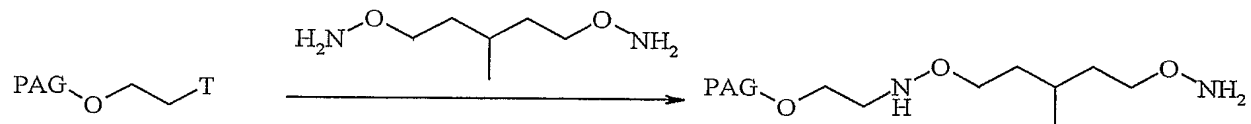
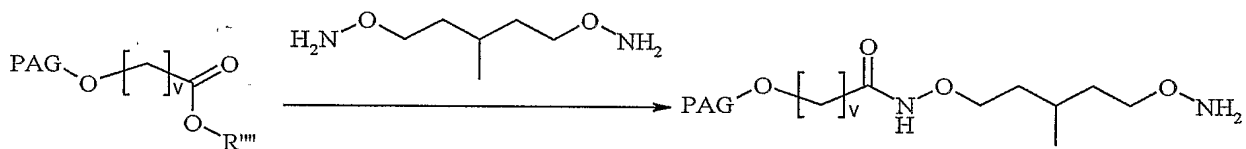
Scheme 1b: polyalkylene glycol (PAG)-derivatives



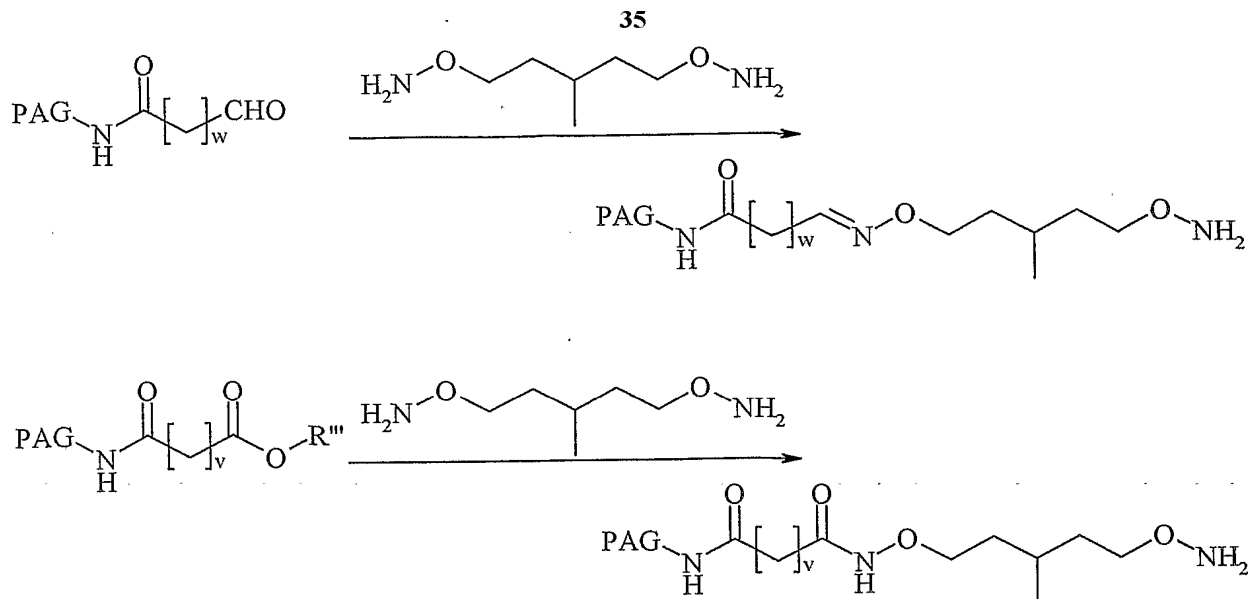
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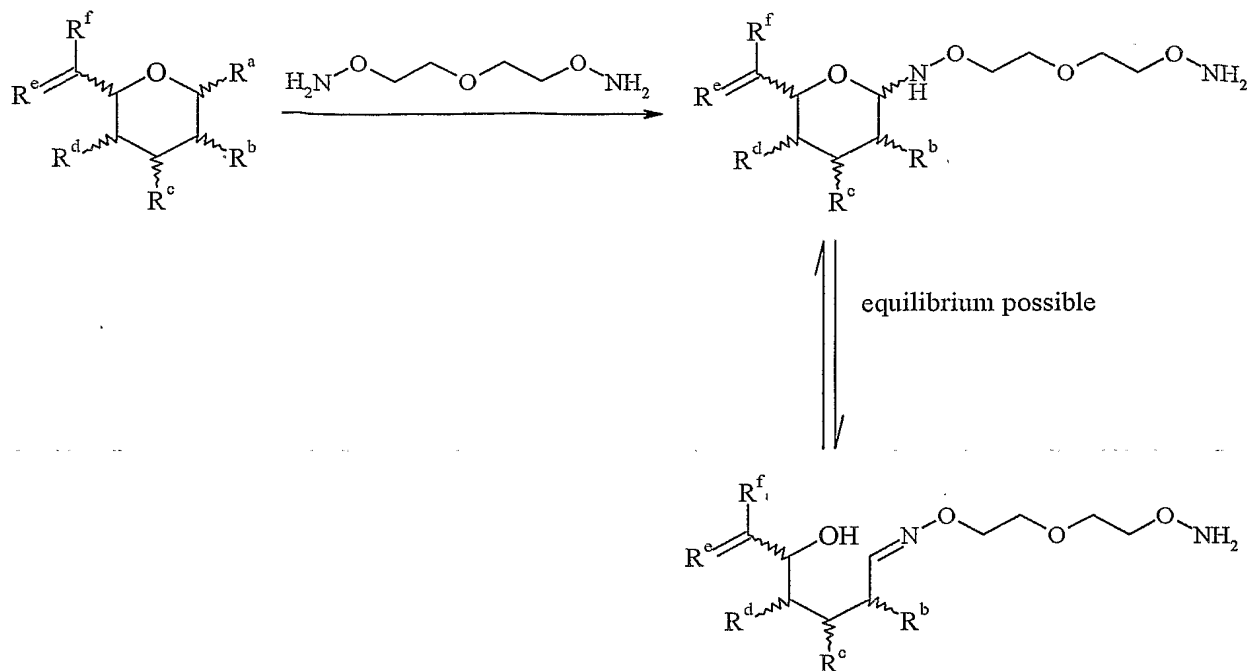


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- 5 wherein OR''' , OR'''' and OR''''' are derived from alcohols $\text{H}-\text{OR}'''$, $\text{H}-\text{OR}''''$ and $\text{H}-\text{OR}'''''$ said alcohols preferably being selected from the group consisting of N-hydroxy succinimides such as N-hydroxy succinimide or Sulfo-N-hydroxy succinimide, suitably substituted phenols such as p-nitrophenol, o,p-dinitrophenol, o,o'-dinitrophenol, trichlorophenol such as 2,4,6-trichlorophenol or 2,4,5-trichlorophenol, trifluorophenol such as
- 10 2,4,6-trifluorophenol or 2,4,5-trifluorophenol, pentachlorophenol, pentafluorophenol, or hydroxyazoles such as hydroxy benzotriazole. Especially preferred are N-hydroxy succinimides, with N-hydroxy succinimide and Sulfo-N-hydroxy succinimide being especially preferred, v is 1 to 10, preferably 1 to 5, more preferably 2 or 3; T is Cl, Br, I or OTf, and PEG is polyethylene glycol or a derivative thereof, preferably mPEG. PAG is
- 15 polyalkylene glycol or a derivative thereof, preferably PEG, more preferably mPEG. w is 1 to 10, preferably 1 to 5, more preferably 4.

Scheme 2: Reaction of the reducing end of polysaccharides



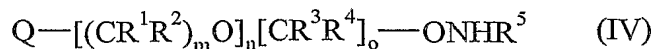
wherein R^e is 2H or O, R^a is OH, R^b , R^c , R^d , R^f are independently H, OH, O-alkyl, hydroxyalkyl, $\text{OC}(\text{O})\text{R}^{\text{''''''}}$, wherein $\text{R}^{\text{''''''}}$ is alkyl, preferably O-acetyl, OPO_3H_2 , OSO_3H ,
 5 ONO_2 or O-polysaccharide; e.g. for dextran and derivatives thereof R^f is O-polysaccharide and for starch and derivatives thereof R^d is O-polysaccharide.

A further embodiment of the present invention relates to polymers as obtainable by a process as mentioned before. Preferred embodiments of the process and the starting materials used are
 10 also mentioned before.

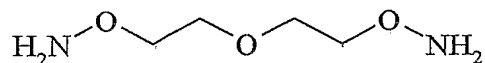
The functionalized polymer obtainable by the process mentioned above is preferably the functionalized polymer of formula I.

15 The functionalized polymers of the present invention are suitable as starting materials for the preparation of a conjugate of the functionalized polymer and a protein. In the conjugate a polymer selected from soluble linear or branched homopolymers or random copolymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, preferably ethylene glycol homopolymers (PEG), propylene glycol homopolymers alkylene
 20 glycol copolymers, preferably propylene oxide/ethylene oxide co-polymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids and polysaccharides, preferably selected from the

group consisting of starch, cellulose, dextran, gum arabic, xanthan gum, inulin, ghatti gum, pectin, guar gum, gum tragacanth, agar, algin, karaya gum, carrageenan, scleroglucan, fucellaran, arabinogalacton and locust bean gum, is covalently linked by a linking group of formula (IV)

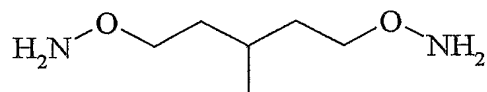


preferably by a linking group of the following formula

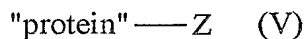


with a protein as mentioned above. Preferred polymers, linking groups and proteins are mentioned before.

In a further preferred embodiment the linking group has the formula (IV)



In a further embodiment the present invention relates to a process for preparing a conjugate, comprising the step of reacting a functionalized polymer of the present invention with a functionalized protein of formula V



wherein Z is a group comprising a carbonyl group or a group which is suitable of forming a carbonyl group or another group which is reactable with the functionalized polymer, wherein Z is covalently linked with least one terminal group or least one centrally located group of the "protein", preferably with an oxidized N-terminal amino acid or an oxidized carbohydrate side chain of the "protein". Preferred functionalized polymers and proteins are mentioned before.

According to one embodiment of the present invention, the functional group Z of the protein is a group which is reactable with the polymer functionalized by a linking group having the reactable end group $-ONHR^5$ (preferably the functionalized polymer of formula I), wherein R^5 is hydrogen, alkyl or aryl, preferably hydrogen. Preferred groups Z are therefore aldehyde

groups, keto groups, carboxy groups, and activated carboxy groups, for example ester groups, and lactone groups. Further suitable groups Z are halide or pseudo halide groups and the like, for example Cl, Br, I, or OTf. Preferably Z is an aldehyde group or a keto group. Therefore, the present invention relates to a method and conjugates as described above, wherein the
5 functional group Z of the protein is an aldehyde group or a keto group.

While there are no general restrictions as to the location of the group Z, preferably the aldehyde or keto group within the protein, the aldehyde or keto group is, according to a preferred embodiment of the present invention, located in a carbohydrate side chain of the
10 protein. Therefore, in the context of this embodiment, a glycosylated protein is employed.

In the context of the present invention, the term "carbohydrate side chain" refers to oligosaccharide connected covalently to an amino acid of a protein and consisting of at least two "carbohydrate moieties". In the context of the present invention the term "carbohydrate
15 moieties" refers to hydroxyaldehydes or hydroxyketones as well as to chemical modifications thereof (see Römpp Chemielexikon, Thieme Verlag Stuttgart, Germany, 9th edition 1990, Volume 9, pages 2281-2285 and the literature cited therein). Furthermore, it also refers to derivatives of naturally occurring carbohydrate moieties like glucose, galactose, mannose, sialic acids and the like.

20 In an even more preferred embodiment, the aldehyde group or the keto group is a galactose residue of the carbohydrate side chain, preferably the terminal galactose residue of the carbohydrate side chain.

25 Oxidation of terminal carbohydrate moieties can be performed either chemically or enzymatically.

Methods for the chemical oxidation of carbohydrate moieties of polypeptides are known in the art and include the treatment with periodate (Chamow et al., 1992, J. Biol. Chem., 267,
30 15916-15922).

By chemically oxidizing, it is in principle possible to oxidize any carbohydrate moiety, being terminally positioned or not. However, by choosing mild reaction conditions it is possible to

preferably oxidize the terminal sialic acid of a carbohydrate side chain to give the aldehyde group or the keto group.

According to a further embodiment of the present invention, said mild reaction conditions
5 relate to reacting the protein with a suitable aqueous periodate solution, having a preferred periodate concentration in the range of from 1 to 50 mM, more preferably of from 1 to 25 mM and especially preferably of from 1 to 10 mM such as about 1mM, and at a preferred reaction temperature of from 0 to 40°C and especially preferably of from 0 to 21 °C such as about 0 °C, and for a preferred reaction time of from 5 min to 5 h, more preferably from 10 min to 2 h
10 and especially preferably from 10 min. to 1 h such as about 1 h. The preferred molar ratio of periodate: protein is from 1 : 200 to 1 : 1 and more preferably from 1 : 50 to 1 : 5, such as about 1:15

If e.g., EPO is chemically oxidized, it is preferred to react EPO in an aqueous medium,
15 preferably water, with periodate solution for preferably 1 h, at a molar ratio of periodate : EPO of about 15 : 1, a reaction temperature of about 0 °C, using an aqueous periodate solution having a concentration of about 1 mM.

Therefore, the present invention also relates to a method and a conjugate as described above,
20 wherein, prior to the reaction of the protein and the polymer or polymer derivative, a glycosylated protein is reacted with a periodate solution to give a protein having an aldehyde group or a keto group located in the oxidized carbohydrate side chain, said reaction preferably being carried out at mild oxidation reactions. The term "mild reaction conditions" as used in this context refers to, e.g., to a 1 mM periodate solution and a reaction temperature of 0 °C in
25 contrast to harsh conditions such as a 10 mM periodate solution and a reaction temperature of 20 to 25 °C.

Alternatively, the carbohydrate side chain may be oxidized enzymatically. Enzymes for the oxidation of the individual carbohydrate side chain are known in the art, e.g. in the case of
30 galactose the enzyme is galactose oxidase. If it is intended to oxidize terminal galactose moieties, it will be eventually necessary to remove terminal sialic acids (partially or completely) if the polypeptide has been produced in cells capable of attaching sialic acids to carbohydrate chains, e.g. in mammalian cells or in cells which have been genetically modified to be capable of attaching sialic acids to carbohydrate chains. Chemical or enzymatic methods

for the removal of sialic acids are known in the art (Chaplin and Kennedy (eds.), 1996, Carbohydrate Analysis: a practical approach, especially Chapter 5 Montreuil, Glycoproteins, pages 175-177; IRL Press Practical approach series (ISBN 0-947946-44-3)).

5 According to another preferred embodiment of the present invention, the aldehyde group or keto group may be located at the N terminus of the protein and is accessible by suitable oxidation. Especially in the case that a hydroxy group containing amino acid is located at the N terminus of the protein at position -1, such as threonine or serine, oxidation of said N-terminal amino acid can be carried out leading to said keto group or an aldehyde group, preferably an aldehyde group. Threonine, e.g., is preferably located at the N terminus of the protein which is an expression product, e.g. produced in eukaryotic cells such as mammalian, especially human, insect or yeast cells, and which is glycosylated with mammalian or other eukaryotic carbohydrates. As method for the chemical oxidation of the suitable N-terminal amino acid, any conceivable method may be applied, with the oxidation with periodate being
10 preferred, with mild oxidation conditions being especially preferred.
15

According to a further preferred embodiment of the present invention, said mild reaction conditions, with regard to the N terminal amino acid, relate to reacting the protein with a suitable aqueous periodate solution, having a preferred periodate concentration in the range of
20 from 1 to 50 mM, more preferably of from 1 to 25 mM and especially preferably of from 1 to 10 mM such as about 1mM, and at a preferred reaction temperature of from 0 to 40 °C and especially preferably of from 0 to 21 °C such as about 0 °C, and for a preferred reaction time of from 5 min to 5 h, more preferably from 10 min to 2 h and especially preferably from 10 min. to 1 h such as about 1 h. The preferred molar ratio of periodate : protein is from 1 : 200
25 to 1 : 1 and more preferably from 1 : 50 to 1 : 5 such as about 15 : 1.

Therefore, the present invention also relates to a method and a conjugate as described above, wherein the aldehyde group or the keto group is located in a carbohydrate side chain of the protein and/or at the N-terminal group of the protein.
30

In a preferred embodiment of the process for preparing the conjugate of the functionalized polymer and the functionalized protein of formula V to an aqueous solution of the functionalized protein, preferably in a sodium acetate buffer at a pH of 5.0 to 5.5, was added

an aqueous solution of the functionalized polymer, preferably in a sodium acetate buffer at a pH of 5.0 to 5.5 usually at a temperature of from 0 to 40 °C, preferably of from 0 to 25 °C, more preferably of from 15 to 25 °C. The solution is then usually incubated in general at the temperature mentioned before. The incubation is usually carried out for 3 to 72 h, preferably 8 to 48 h, more preferably 15 to 25 h. The steps for work up and isolation of the conjugate are known by a person skilled in the art. The conjugate may be subjected to a further treatment such as an after-treatment like dialysis, centrifugal filtration or a pressure filtration, ion exchange chromatography, reversed phase chromatography, HPLC, MPLC, gel filtration and/or lyophilization.

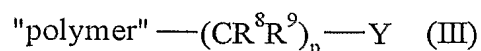
The molar ratio of the functionalized polymer of formula III and the functionalized protein of formula V is usually of from 1-50 : 1, preferably of from 1-30 : 1, more preferably of from 1-20 : 1, even more preferably of from 1-15 : 1, and most preferably of from 1-5 : 1, when the functionalized polymer of formula III is precipitated by adding an alcohol, preferably 2-propanol or ethanol.

The molar ratio of the functionalized polymer of formula III and the functionalized protein of formula V is usually of from 1-200 : 1, preferably of from 1-100 : 1, more preferably of from 1-50 : 1, when the functionalized polymer of formula III is precipitated by adding a solvent mixture of ethanol and acetone.

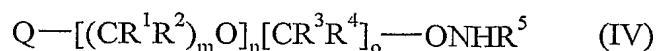
It is further possible to prepare the conjugate of the present invention in a two step process starting from a functionalized polymer of formula III.

In a further embodiment the present invention therefore relates to a process for preparing a conjugate, comprising the steps

a) reacting a polymer of formula III



with a compound of formula IV



wherein the symbols have the following meanings

“polymer” soluble linear or branched homopolymers or random copolymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, preferably ethylene glycol homopolymers (PEG), propylene glycol homopolymers, alkylene glycol copolymers, preferably propylene oxide/ethylene oxide co-polymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids and polysaccharides, preferably selected from the group consisting of starch, cellulose, dextran, gum arabic, xanthan gum, inulin, ghatti gum, pectin, guar gum, gum tragacanth, agar, algin, karaya gum, carrageenan, scleroglucan, fucellaran, arabinogalacton and locust bean gum;

$R^1, R^2, R^3,$

R^4, R^5 hydrogen, alkyl, aryl, preferably hydrogen;

m 2 to 4, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2 ;

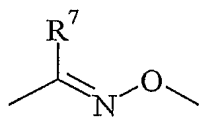
n 0 to 20, preferably 0 to 10, more preferably 1 to 5, most preferably 1 or 2, and even more preferably 1;

o 0 to 20, preferably 0 to 10, more preferably 0 or 2, wherein in the case of $n = 0$, o is not 0,

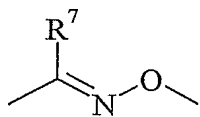
in a preferred embodiment o is 2 to 20, preferably 2 to 10, more preferably 2,

wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4 ;

Y and Q functional groups, which are suitable to react together to give one of the following linking groups -O-, -S-, -NR⁶-, -OC(O)-, -C(O)O-, -C(G)N(R¹⁰)O-, -N(R¹¹)O-,



, preferably



, -N(R¹¹)O-, -C(G)N(R¹⁰)O-,

wherein one or more groups $-(CR^8R^9)-$ may be replaced by W, whereby a chemically reasonable group is formed;

W O, NR^{12} , C(G), preferably O, C(G);

G S, O, NR^{14} , preferably O;

$R^6, R^7, R^8,$

$R^9, R^{10},$

R^{11}, R^{12}, R^{14} hydrogen, alkyl, aryl, preferably hydrogen;

p 0 to 20, preferably 0 to 10, more preferably 0 to 5, most preferably 0 to 4, even more preferably 0 in the case of polysaccharides and derivatives thereof, and 1 to 4 in the case of polyalkylene glycols and derivatives thereof, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 ;

wherein the group

$-(CR^8R^9)_p-Y$

is covalently linked with least one terminal group and/or least one centrally located group of the "polymer", wherein a functionalized polymer is obtained, and

b) reacting the functionalized polymer obtained in step a) with a functionalized protein of formula V

"protein" — Z (V)

wherein Z is a group comprising a carbonyl group or a group which is suitable of forming a carbonyl group or another group which is reactable with the functionalized polymer, wherein Z is covalently linked with least one terminal group and/or least one centrally located group of the "protein", preferably with an oxidized N-terminal amino acid or an oxidized carbohydrate side chain of the "protein". Preferred polymers of formula III, compounds of formula IV and functionalized proteins of formula V are the same as mentioned before.

The reaction conditions of steps a) and b) are the same as mentioned for the preparation of the functionalized polymer, preferably the polymer of formula I (step a)) and for the preparation of the conjugate starting from the functionalized polymer (step b)). The advantage of the two step process is that the isolation step of the functionalized polymer obtained in step a) may be omitted.

The conjugates of the present invention themselves or a pharmaceutical composition comprising the conjugates are useful in a method for a treatment of the human or animal body.

5

In a further embodiment the present invention relates to the conjugate of the present invention, or the conjugate, obtainable by a method of the present invention, for use in a method for the treatment of the human or animal body.

10 The pharmaceutical compositions comprising a therapeutically effective amount of the conjugate of the present invention as well as pharmaceutically acceptable diluent, adjuvant or carrier. The pharmaceutical compositions further optionally comprising further therapeutical or galenic components and adjuvants. Suitable adjuvants are for example diluents, buffer systems, binders, surface active components, thickening agents, lubricants and antidegradants
15 (enclosing antioxidants).

A therapeutically effective amount is the amount which is sufficient to achieve a positive effect in a singular or repeatedly treatment within the scope of a treatment for facilitation, healing or prevention of a disease.

20

A pharmaceutically acceptable diluent is a diluent which is compatible with both, the conjugate of the present invention and the human or animal body.

The form of the pharmaceutical composition is depending on the desired or suitable way of
25 application. The preferred application is the parenteralic application. Suitable parenteralic applications are known in the art. Further possible applications are the intranasalic, intrachealic, or topic application. The pharmaceutical compositions may be presented in form of a dose unit and are prepared by a process known in the art.

30 A pharmaceutical composition comprising in a therapeutically effective amount the conjugate of the present invention, or the conjugate, obtainable by a method of the present invention.

Preferably the pharmaceutical composition further comprising at least one pharmaceutically acceptable diluent, adjuvant, or carrier. Suitable diluents, adjuvants, or carriers as well as further suitable ingredients are known by a person skilled in the art.

- 5 The following examples additionally describe the present invention.

Examples**A) Synthesis of hydroxylamino-PEG-derivatives****5 Aa) Synthesis of Hydroxylamino-PEG20 in aqueous solution**

200 mg of mPEG-Butyraldehyde (mPEG-ButyrALD, MW20,000, Nektar, Huntsville, AL, USA) were dissolved in 2 mL 0.1M sodium acetate buffer, pH 5.2 and 1 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine (synthesized according to D. Boturn et al.,
10 Tetrahedron 53 (1997) 5485-92, pp. 5489-90) were added. After shaking for 19 h at 22°C, the reaction mixture was added to 45 mL 2-propanol at -20°C and incubated at -20°C for 4 h. The precipitated product was collected by centrifugation at 0°C, washed with 15 mL 2-propanol at -20°C and incubated at -20°C for 1 h. After centrifugation, the product was dissolved in 15 mL water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio
15 Sciences Deutschland GmbH, Bonn, D) and lyophilized.

Ab) Synthesis of Hydroxylamino-PEG20 in organic solvents

200 mg of mPEG-Butyraldehyde (mPEG-ButyrALD, MW20,000, Nektar, Huntsville, AL, USA) were dissolved in 2 mL dichloromethane and 1 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine (synthesized according to D. Boturn et al., Tetrahedron 53 (1997) 5485-92, pp. 5489-90) were added. After shaking for 19 h at 22°C, the reaction mixture was added to 45 mL 2-propanol at -20°C and incubated at -20°C for 4 h. The precipitated product was collected by centrifugation at 0°C, washed with 15 mL 2-propanol at -20°C and
25 incubated at -20°C for 1 h. After centrifugation, the product was dissolved in 15 mL water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

B) Synthesis of a hydroxylamino-dextran-derivate

30

Synthesis of Hydroxylamino-Dextran17 in aqueous solution

500 mg of Dextran (Dextran from Leuconostoc ssp., M_r ~15000-20000D, Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) were dissolved in 5 mL 0.1M sodium acetate buffer,

pH 5.2 and 1 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine (synthesized according to D. Boturn et al., Tetrahedron 53 (1997) 5485-92, pp. 5489-90) were added. After shaking for 19 h at 22°C, the reaction mixture was added to 45 mL 2-propanol at -20°C and incubated at -20°C for 4 h. The precipitated product was collected by centrifugation at 0°C, washed with 15 mL 2-propanol at -20°C and incubated at -20°C for 1 h. After centrifugation, the product was dissolved in 15 mL water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

10 C) Synthesis of the EPO conjugates

C1) Periodate oxidation of N-acetylaneuraminic acid residues by mild periodate treatment of EPO

15 To a 2,0 mg/ml solution of EPO (recombinantly produced EPO having amino acid sequence of human EPO and similar or essentially the same characteristics as the commercially available (Epoietin alpha :Erypo, ORTHO BIOTECH, Jansen-Cilag or Epoietin beta: NeoRecormon, Roche; cf. EP 0 148 605, EP 0 205 564, EP 0 411 678) of total 20ml kept at 0°C were added 2,2ml of an ice-cold solution of 10mM sodium meta-periodate resulting in a final concentration of 1mM sodium meta-perjodate. The mixture was incubated at 0°C for 1 hour in an ice-bath in the dark and the reaction was terminated by addition of 40µl of glycerol and incubated for further 5 minutes.

25 C2) Buffer exchange of periodate oxidised EPO for subsequent derivatisation with a hydroxylamino functionalized hydroxyethyl starch derivative

Buffer exchange was performed using a 20 ml Vivaspin 20 concentrator (Vivaspin AG, Hannover, Germany) with a polyethersulfone (PES) membrane. The concentrator unit was washed by addition of 5 ml of 0.1 M Na-acetate buffer pH 5.5 and centrifugation of the concentrator unit at 4000 rpm at 6°C in a Megafuge 1.0R (Kendro Laboratory Equipment, Osterode, Germany). Subsequently, 20 ml of the perjodate oxidised EPO solution according to Example 1 was added to the concentrator unit and was centrifuged at 4000 rpm for 25min until a 5-fold concentration was achieved. 15 ml of 0.1 M Na-acetate buffer pH 5.5 was added

to the concentrate which was centrifuged as described above. The centrifugation cycle was repeated 3 times, the final concentrate was removed and transferred into a 50 ml sterile plastic tube, after washing of the concentrator unit 2 times with each 1 ml of Na-acetate buffer pH 5.5; the volume of the EPO was adjusted with Na-acetate buffer pH 5.5 to 26.7 ml and protein concentration of the final oxidised EPO solution was determined by measuring the absorbance at 280 nm using the specific absorbance value of 7.43 as described in the European Pharmacopeia (Erythropoietin Concentrated Solution, 4th Edition, 2002, pages 1123-1128). A value of 1.378 mg / ml was determined for the final periodate oxidised EPO solution (36.8 mg EPO, corresponding to ≈ 90 % final yield).

C3) Synthesis of the EPO-conjugates of mPEG aldehyde-reactive polymer and dextran aldehyde-reactive polymer

To 13.3 μ L of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (oxidation according to example C 1), 6.67 μ L of a solution of the aldehyde-reactive polymer in 0.1 M sodium acetate buffer, pH 5.5 were added and the solution was incubated for 22 h at 22°C. The following concentrations were employed:

100 mg/mL for aldehyde-reactive polymer prepared according to example Aa) and Ab)

87.5 mg/mL for dextran aldehyde-reactive polymer prepared according to example B)

In Examples C1) to C3), a successful conjugation is indicated by the migration of the protein bands to higher molecular weights in the SDS page analysis according to Figure 2. The increased band-width is due to the molecular weight distribution of the dextran and PEG-derivatives used and the number of HES derivatives linked to the protein. In contrast to the hydroxylamino-dextran derivative, the corresponding PEG derivative migrates into the gel as well and is also stained, therefore complicating the visualisation of the protein bands.

C4) Synthesis of conjugates of hydroxyethyl starch and EPO

C4.1) Synthesis of hydroxylamino functionalized HES derivatives

O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine was synthesized as described in Boturyn et al. Tetrahedron 53 (1997) p. 5485-5492 in 2 steps from commercially available materials.

C4.1) (a) Synthesis of hydroxylamino-HES 10 / 0.4

2 g of HES10/0.4 (MW = 10000 D, DS = 0.4, Supramol Parenteral Colloids GmbH, Rosbach-
5 Rodheim, D) were dissolved in 17 mL 0.1M sodium acetate buffer, pH 5.2 and 20 mmol O-
[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for 19 h at 22°C,
the reaction mixture was added to 100 mL of an ice-cold 1:1 mixture of acetone and ethanol
(v/v). The precipitated product was collected by centrifugation at 4°C, re-dissolved in 50 mL
water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio
10 Sciences Deutschland GmbH, Bonn, D) and lyophilized.

C4.1) (b) Synthesis of hydroxylamino-HES 10 / 0.7

2 g of HES10/0.7 (MW = 10000 D, DS = 0.7, Supramol Parenteral Colloids GmbH, Rosbach-
15 Rodheim, D) were dissolved in 18 mL 0.1M sodium acetate buffer, pH 5.2 and 20 mmol O-
[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for 19 h at 22°C,
the reaction mixture was added to 100 mL of an ice-cold 1:1 mixture of acetone and ethanol
(v/v). The precipitated product was collected by centrifugation at 4°C, re-dissolved in 50 mL
water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio
20 Sciences Deutschland GmbH, Bonn, D) and lyophilized.

C4.1) (c) Synthesis of hydroxylamino-HES 50 / 0.4

2 g of HES50/0.4 (MW = 50000 D, DS = 0.4, Supramol Parenteral Colloids GmbH, Rosbach-
25 Rodheim, D) were dissolved in 20 mL 0.1M sodium acetate buffer, pH 5.2 and 4 mmol O-[2-
(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for 19 h at 22°C, the
reaction mixture was added to 100 mL of an ice-cold 1:1 mixture of acetone and ethanol
(v/v). The precipitated product was collected by centrifugation at 4°C, re-dissolved in 50 mL
water, dialysed for 21 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio
30 Sciences Deutschland GmbH, Bonn, D) and lyophilized.

C4.1) (d) Synthesis of hydroxylamino-HES 50 / 0.7

2 g of HES50/0.7 (MW = 50000 D, DS = 0.7, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were dissolved in 20 mL 0.1M sodium acetate buffer, pH 5.2 and 4 mmol O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine were added. After shaking for 17.5 h at 22°C, the reaction mixture was added to 70 mL of an ice-cold 1:1 mixture of acetone and ethanol (v/v). The precipitated product was collected by centrifugation at 0°C, washed with 30 mL of an ice-cold 1:1 mixture of acetone and ethanol (v/v), re-dissolved in 50 mL water, dialysed for 19.5 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

C4.2) Synthesis of HES-EPO conjugates

In Examples C4.2) (a) to C4.2) (d), a successful conjugation is indicated by the migration of the protein bands to higher molecular weights in the SDS page analysis according to Figure 1. The increased band-width is due to the molecular weight distribution of the HES derivatives used and the number of HES derivatives linked to the protein.

C4.2) (a) Synthesis with of hydroxylamino-HES 10 / 0.4 according to Example C4.1) **(a)**

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example C2); 1.378 mg/ml), 83 mg of hydroxylaminoHES10/0.4, produced according to example C4.1) (a), were added and the solution was shaken for 16.5 h at 22°C.

C4.2) (b) Synthesis with of hydroxylamino-HES 10 / 0.7 according to Example C4.1) **(b)**

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example C2); 1.378 mg/ml), 83 mg of hydroxylaminoHES10/0.7, produced according to example C4.1) (b), were added and the solution was shaken for 16.5 h at 22°C.

C4.2) (c) Synthesis with of hydroxylamino-HES 50 / 0.4 according to Example C4.1) **(c)**

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example C2); 1.378 mg/ml), 416 mg of hydroxylaminoHES50/0.4, produced according to example C4.1) (c), were added and the solution was shaken for 16.5 h at 22°C.

C4.2) (d) Synthesis with of hydroxylamino-HES 50 / 0.7 according to Example C4.1)

To 3.63 mL of a solution of oxidized EPO in 0.1 M sodium acetate buffer, pH 5.5 (according to Example C2); 1.378 mg/ml), 416 mg of hydroxylaminoHES50/0.7, produced according to example C4.1) (d), were added and the solution was shaken for 16.5 h at 22°C.

D) Purification of HES-modified EPO and separation of unreacted HES-derivatives from HES-modified EPO

Subsequent to the HES-coupling procedures according to Examples C4.2) (a) to C4.2) (d), the
15 purification of all samples was performed at room temperature using an ÄKTA explorer 10
system equipped with a Pump P-903, Mixer M-925 with 0.6 ml chamber, Monitor pH/C-900,
pump P-950 (sample pump) along with a Software Unicorn Version 3.21. Detection was at
280, 260 and 220 nm using a Monitor UV-900 with a 10 mm flow cell.

20 The incubation mixtures were diluted with 10 volumes of buffer A (20 mM N-morpholino propane sulfonic acid adjusted to pH 8.0 with NaOH) and were applied to a column containing 4 ml Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) at a flow rate of 0.8 ml/min; the column was previously equilibrated with 7 column volumes (CV) of buffer A. The column was then washed with 6 CV of buffer A at a flow rate of 1.0 ml/min and elution
25 was performed by using 2.5 CV of buffer B (0.5 M NaCl in 20 mM Na-phosphate, pH 6.5) at a flow rate of 0.6 ml/min. The column was then washed with 2.5 CV of buffer C (1.5 M NaCl in 20 mM Na-phosphate, pH 6.5) at a flow rate of 0.6 ml/min and was re-equilibrated by passing 7 CV of buffer A at flow rate of 1.0 ml/min.

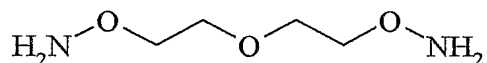
30 Samples from incubations with (activated) hydroxylaminoHES derivatives all yielded significant absorption at 220 nm. The samples incubated with hydroxylaminoHES10/0.4 and 10/0.7, respectively, gave no detectable absorption at 280 nm, whereas the samples incubated with HydroxylaminoHES50/0.7 and 50/0.4, respectively, yielded 800 mAU x ml and 950 mAU x ml, respectively. The bound proteins were recovered in a volume of 6.5 - 8.0 ml

almost exclusively in eluate 1, with eluate 2 containing < 2% of the peak area of totally eluted peaks detected at 280 nm. The protein recovery was comparable for all EPO samples (approximately 85%).

5 HES-modified EPO and EPO from appropriate control incubations were subjected to buffer exchange by using 5 ml Vivaspin concentrators (10,000 MW cut-off) and centrifugation at 4000 rpm at 6°C as described previously. Samples (1-3 mg of EPO protein) were concentrated to 0.5-0.7 ml and were diluted with phosphate buffered saline (PBS) pH 7.1 to 5 ml and subjected to 10-fold concentration by centrifugation. Each sample was subjected to the
10 concentration and dilution cycle three times. Finally, samples were withdrawn and the concentrator units were washed with 2x 0.5 ml of PBS. Samples were frozen in liquid nitrogen at protein concentrations of approximately 1.2 mg/ml.

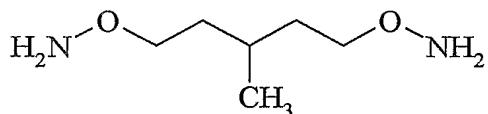
15 E) Synthesis of Crosslinking Compounds

E1) Synthesis of O-[2-(2-aminooxy-ethoxy)-ethyl]-hydroxyl amine (1)



Compound (1) is synthesized according to D. Boturny et al., Tetrahedron 53 (1997) 5485-92,
20 pp. 5489-90, as already described in example Aa) hereinabove.

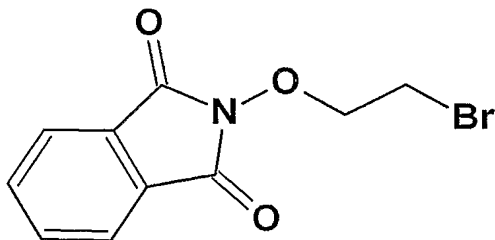
E2) Synthesis of 1,6-Bis-aminooxy-3-methyl pentane (4)



20 g (82.0 mmol) 1,5-dibromo-4-methylpentane (Acros Organics BVBA, Geel, B) were
25 added drop-wise at room temperature to a suspension of 36.72 g (204.9 mmol) endo-N-hydroxy-5-norbornene-2,3-dicarboxamide (Lancaster Synthesis GmbH, Frankfurt/Main, D) and 36.7 g potassium carbonate (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) in 140 ml N,N-dimethylformamide (DMF) (Peptide synthesis grade, Biosolve, Valkenswaard, NL), and the reaction mixture was stirred for 90 h. The fine precipitate was removed by
30 centrifugation, and the clear DMF supernatant was collected and concentrated *in vacuo*. The crude product was dried thoroughly *in vacuo*, dissolved in 140 ml ethanol (DAB quality, Sonnenberg, Braunschweig, D) and refluxed under nitrogen together with 16.2 ml (333.3

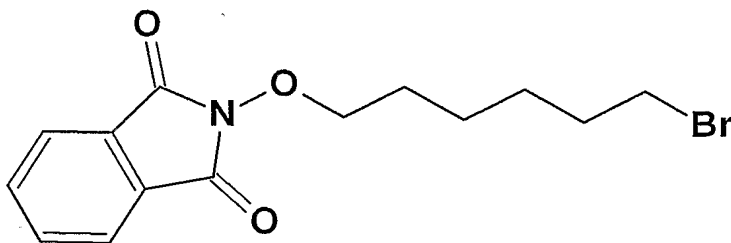
mmol) hydrazine hydrate (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) for 2 h. The solvent and remaining hydrazine hydrate was removed *in vacuo* and the crude product was suspended in 150 ml tert-butyl methyl ether (MTBE) (Acros Organics BVBA, Geel, B) and stirred for 1 h at room temperature. The precipitate was filtered off, washed with MTBE and discarded. The collected filtrates were concentrated *in vacuo* and the product was purified by column chromatography (3 % methanol in dichloromethane, both Acros Organics BVBA, Geel, B) yielding 38% of (**4**) as colourless oil. δ_{H} (300 MHz; CDCl_3) 0.89 (3 H, d, J 6.5), 1.37 – 1.44 (2 H, m), 1.55-1.66 (3 H, m), 3.64 - 3.71 (4 H, m), 5.30 (4H, s); δ_{C} (75.5 MHz; CDCl_3) 19.7, 27.2, 35.4, 74.3.

E3) Synthesis of 2-(2-Bromoethoxy)-3a,7a-dihydro-isoindole-1,3-dione (5**)**



Compound (**5**) was synthesized as described in Bauer, L. and Suresh, K. S., J. Org. Chem. 1963, 28, p. 1604, from 16.3 g (100 mmol) N-hydroxyphthalimide (Acros Organics BVBA, Geel, B), 17.0 ml (196.9 mmol) 1,2-dibromoethane (Lancaster Synthesis GmbH, Frankfurt/Main, D) and 28 ml (197.6 mmol) triethylamine (Acros Organics BVBA, Geel, B) in 120 ml DMF (Peptide synthesis grade, Biosolve, Valkenswaard, NL). After removing of the formed precipitate by filtration, the crude product was precipitated with 800 ml water, collected by filtration and purified by column chromatography (25 % tert-butyl methyl ether in petroleum ether, both Acros Organics BVBA, Geel, B) yielding 7.58 g (29%) of (**5**) as an off white solid. δ_{H} (400 MHz; CDCl_3) 3.63 (3 H, t, J 6.9), 4.46 (3 H, t, J 6.9), 7.75 (2 H, dd, J 3.1 and 5.5), 7.83 (2 H, dd, J 3.1 and 5.5).

E4) Synthesis of 2-(6-Bromo-hexyloxy)-3a,7a-dihydro-isoindole-1,3-dione (6**)**



Compound (**6**) was synthesized as described in Bauer, L. and Suresh, K. S., J. Org. Chem. 1963, 28, p. 1604, from 16.3 g (100 mmol) N-hydroxyphthalimide (Acros Organics BVBA, Geel, B), 30.8 ml (200 mmol) 1,6-dibromohexane (Aldrich, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) and 28 ml (197.6 mmol) triethylamine (Acros Organics BVBA, Geel, B) in 120 ml DMF (Peptide synthesis grade, Biosolve, Valkenswaard, NL). Purification by column chromatography (10 % tert-butyl methyl ether in petroleum ether, both Acros Organics BVBA, Geel, B) yielded 12.28 g (38%) of (**6**) as an off white solid. δ_H (400 MHz; $CDCl_3$) 1.48 - 1.60 (4 H, m), 1.77 - 1.84 (2 H, m), 1.87 - 1.93 (2 H, m), 3.42 (2 H, t, J 6.8), 4.20 (2 H, t, J 6.5), 7.71 - 7.76 (2 H, m), 7.81 - 7.85 (2 H, m); δ_C (100.6 MHz; $CDCl_3$) 24.9, 27.9, 28.0, 32.7, 33.7, 78.4, 123.5, 129.1, 134.5, 163.7.

F) Preparation of reactive Polymer-Derivatives of the Hydroxylamine Crosslinker

15 F1) Synthesis of Hydroxylamino-Dextran17.5 (**A**) from Crosslinker (**4**) and Dextran 15-20kD.

204 mg of Dextran 15-20kD (MW = 15000 - 20000 D, Fluka, Sigma-Aldrich Chemie, Taufkirchen, D) were dissolved in 2 mL 0.1M sodium acetate buffer, pH 5.2 and 0.57 mmol of (**4**) were added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL of ice-cold 2-propanol and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL 2-propanol and again collected by filtration. The crude product was dissolved in 15 ml water, dialysed for 43.5 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 82%.

F2) Synthesis of Hydroxylamino-HES10/0.4 (**B**) from Crosslinker (**4**) and HES10/0.4

403 mg of HES10/0.4 (MW = 10000 D, DS = 0.4, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were dissolved in 4 mL 0.1M sodium acetate buffer, pH 5.2 and 4 mmol of (**4**) were added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL of ice-cold 2-propanol and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL

2-propanol and again collected by filtration. The crude product was dissolved in 15 ml water, dialysed for 46 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 52%.

5

F3) Synthesis of Hydroxylamino-PEG10 (C) from Crosslinker (1) and mPEG-NHS ester 10 kD.

10

607 mg of α -methoxy-PEG- ω -succinimidyl ester (MW = 10000 D, Rapp Polymere, Tübingen, D) were dissolved in 6 mL dichloromethane (Acros Organics BVBA, Geel, B) and 3.2 mmol of 1 were added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL of ice-cold 2-propanol and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL 2-propanol and again collected by filtration. The crude product was dissolved in 15 ml

15 water, dialysed for 43.5 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 82%.

20

200 mg of this product was dissolved in 2 ml dichloromethane (Acros Organics BVBA, Geel, B) and 0.2 mmol morpholine (Acros Organics BVBA, Geel, B) was added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL tert-butyl methyl ether (Acros Organics BVBA, Geel, B) and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL tert-butyl methyl ether and again collected by filtration. The crude product was

25 dissolved in 10 ml water, dialysed for 45 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 79%.

25

30

F4) Synthesis of Hydroxylamino-PEG10 (D) from Crosslinker (4) and mPEG-NHS ester 10 kD.

206 mg of α -methoxy-PEG- ω -succinimidyl ester (MW = 10000 D, Rapp Polymere, Tübingen, D) were dissolved in 6 mL dichloromethane (Acros Organics BVBA, Geel, B) and 1 mmol of (4) was added. After shaking for 17 h at 22°C, the reaction mixture

was added to 40 mL of ice-cold 2-propanol and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL 2-propanol and again collected by filtration. The crude product was dissolved in 15 ml water, dialysed for 43.5 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 86%.

100 mg of this product was dissolved in 2 ml dichloromethane (Acros Organics BVBA, Geel, B) and 0.2 mmol morpholine (Acros Organics BVBA, Geel, B) was added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL tert-butyl methyl ether (Acros Organics BVBA, Geel, B) and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL tert-butyl methyl ether and again collected by centrifugation. The crude product was dissolved in 10 ml water, dialysed for 45 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 61%.

F5) Synthesis of HydroxylaminoHES10/0.4 (E) from Crosslinker (4) and oxidized HES10/0.4

400 mg of oxidized HES10/0.4 (MW = 10000 D, DS = 0.4, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were heated at 80°C *in vacuo* for 17 h and dissolved in 4 mL dry DMSO (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D). To the solution 4 mmol (4) were added. After incubation for 5 d at 65°C, the reaction mixture was added to 35 mL of ice-cold 2-propanol and was incubated at -20°C for 1 h. The fine precipitated product was collected by centrifugation at 4°C for 9 h, re-dissolved in 10 mL water, dialysed for 47 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized.

F6) Synthesis of Hydroxylamino-PEG10 (F) from Crosslinker (1) and mPEG-aldehyde 10 kD.

604 mg of α -methoxy-PEG- ω -aldehyde (MW = 10000 D, Rapp Polymere, Tübingen, D) were dissolved in 6 mL 4 mL 0.1M sodium acetate buffer, pH 5.2 and 2.2 mmol of

(1) were added. After shaking for 17 h at 22°C, the reaction mixture was concentrated *in vacuo*, redissolved in 6 ml dichloromethane (Acros Organics BVBA, Geel, B), added to 40 mL of ice-cold 2-propanol and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL 2-propanol and again collected by centrifugation. The crude product was dissolved in 15 ml water, dialysed for 42 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 87%.

10 **F7) Synthesis of Hydroxylamino-PEG10 (G) from Crosslinker (4) and mPEG-aldehyd 10 kD.**

200 mg of α -methoxy-PEG- ω -aldehyde (MW = 10000 D, Rapp Polymere, Tübingen, D) were dissolved in 6 mL 4 mL 0.1M sodium acetate buffer, pH 5.2 and 1 mmol of (4) was added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL of ice-cold 2-propanol and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL 2-propanol and again collected by centrifugation. The crude product was dissolved in 15 ml water, dialysed for 43.5 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 60%.

F8) Synthesis of Hydroxylamino-PEG10 (H) from Crosslinker (6) and mPEG-thiol 10 kD.

300 mg of α -methoxy-PEG- ω -thiol (MW = 10000 D, Rapp Polymere, Tübingen, D) were dissolved in 3 mL N,N-dimethylformamide (DMF) (Peptide synthesis grade, Biosolve, Valkenswaard, NL) and oxygen was removed with a stream of nitrogen. This solution was added under nitrogen to 0.4 mmol of (6) and 0.44 mmol caesium carbonate (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D). After stirring for 2.5 h at 22°C, the reaction mixture was added to 40 mL tert-butyl methyl ether (Acros Organics BVBA, Geel, B) and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL tert-butyl methyl ether, again collected by centrifugation and dried *in vacuo*. The yield of isolated product was not determined.

150 mg of this crude product was dissolved in 1.5 ml dichloromethane (Acros Organics BVBA, Geel, B) and 0.15 mmol hydrazine hydrate (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) were added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL tert-butyl methyl ether and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL tert-butyl methyl ether and again collected by centrifugation. The crude product was dissolved in 10 ml water, dialysed for 41 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 63%.

F9) Synthesis of Hydroxylamino-PEG10 (I) from Crosslinker (5) and mPEG-thiol 10 kD.

300 mg of α -methoxy-PEG- ω -thiol (MW = 10000 D, Rapp Polymere, Tübingen, D) were dissolved in 3 mL N,N-dimethylformamide (DMF) (Peptide synthesis grade, Biosolve, Valkenswaard, NL) and oxygen was removed with a stream of nitrogen. This solution was added under nitrogen to 0.4 mmol of (5) and 0.44 mmol caesium carbonate (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D). After stirring for 2.5 h at 22°C, the reaction mixture was added to 40 mL tert-butyl methyl ether (Acros Organics BVBA, Geel, B) and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL tert-butyl methyl ether, again collected by centrifugation and dried *in vacuo*. The yield of isolated product was not determined.

150 mg of this crude product was dissolved in 1.5 ml dichloromethane (Acros Organics BVBA, Geel, B) and 0.15 mmol hydrazine hydrate (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) were added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL tert-butyl methyl ether and incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL tert-butyl methyl ether and again collected by centrifugation. The crude product was dissolved in 10 ml water, dialysed for 41 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 70%.

F10) Synthesis of HydroxylaminoHES18/0.5 (J) from Crosslinker (1) and oxidized HES18/0.5

200 mg of oxidized HES18/0.5 (MW = 18000 D, DS = 0.5) were heated at 80°C in vacuo for 17 h and dissolved in 2 mL dry DMSO (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D). To the solution 2 mmol of (1) were added. After incubation for 5 d at 65°C, the reaction mixture was added to 20 mL of ice-cold 2-propanol and was incubated at -20°C for 1 h. The precipitated product was collected by centrifugation at 4°C, washed with 42 ml ice-cold 2-propanol, re-dissolved in 10 mL water, dialysed for 27 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 72%.

F11) Synthesis of HydroxylaminoHES10/0.4 (K) from Crosslinker (1) and HES10/0.4

0.8 g of HES10/0.4 (MW = 10000 D, DS = 0.4, Supramol Parenteral Colloids GmbH, Rosbach-Rodheim, D) were dissolved in 8 mL 0.1M sodium acetate buffer, pH 5.5 and 8 mmol of (1) were added. After shaking for 19 h at 22°C, the reaction mixture was added to 40 mL of 2-propanol at -20°C. The precipitated product was collected by centrifugation at 4°C, re-dissolved in 50 mL water, dialysed for 45 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The isolated product yield was 73%.

F12) Synthesis of Hydroxylamino-PEG10 (L) from Crosslinker (6) and mPEG-alcohol 5 kD.

1 g of α -methoxy-PEG-alcohol (MW = 5000 D, Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) and 257 mg powdered potassium hydroxide (Riedel-de Haën, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) were suspended under nitrogen in 10 mL dry dimethyl sulphoxide (DMSO) (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) and the reaction mixture was heated to 70°C. 978 mg of (6) was added as a solid and the mixture was stirring for 2 h at 70°C. The resulting solution was added to 200 mL tert-butyl methyl ether (Acros Organics BVBA, Geel, B) The precipitated product

gum was extracted twice with 100 mL tert-butyl methyl ether, dissolved in 40 ml water, dialysed for 47 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D) and lyophilized. The yield of isolated product was 98%.

5

482 mg of this crude product was dissolved in 5 ml dichloromethane (Acros Organics BVBA, Geel, B) and 1 mmol hydrazine hydrate (Fluka, Sigma-Aldrich Chemie GmbH, Taufkirchen, D) was added. After shaking for 17 h at 22°C, the reaction mixture was added to 40 mL tert-butyl methyl ether and incubated at -20°C for 1 h.

10

The precipitated product was collected by centrifugation at 4°C, suspended in 15 mL tert-butyl methyl ether and again collected by centrifugation. The crude product was dissolved in 10 ml water and dialysed for 41 h against water (SnakeSkin dialysis tubing, 3.5 kD cut off, Perbio Sciences Deutschland GmbH, Bonn, D). Centrifugation for 17 h removed a fine precipitate that formed during dialysis. The clear supernatant was lyophilized, yielding 67% of isolated product.

15

G) Conjugation of the reactive Polymer-Derivatives to oxidized hEPO

20 **G1)** A solution of oxidized hEPO in 0.1 M sodium acetate buffer, pH 5.5 is obtained by a method as described in C1) and C2). The final concentration of oxidised hEPO was adjusted to 2 mg/mL

25 **G2) Synthesis of the EPO conjugates with the polymers prepared in Examples F1) to F12)**

To 20 µL of a solution of oxidized hEPO in 0.1 M sodium acetate buffer, pH 5.5, (as obtained in Example G1), 20 µL of a solution of the aldehyde-reactive polymer in 0.1 M sodium acetate buffer, pH 5.5 were added and the solution was incubated for 22 h at 22°C. The concentrations according to the following Table 1 of the aldehyde-reactive polymers were employed:

30

Table 1

Entry	Polymer Derivative	Synthesized as described in	Equivalents	Concentration [mg/ml]
1	(A)	F1)	5	5.83
2	(B)	F2)	5	3.33
3	(C)	F3)	5	3.33
4	(D)	F4)	5	3.33
5	(E)	F5)	50	33.3
6	(F)	F6)	50	33.3
7	(G)	F7)	50	33.3
8	(H)	F8)	5	3.33
9	(I)	F9)	5	3.33
10	(J)	F10)	50	60.0
11	(K)	F11)	5	3.33
12	(L)	F12)	50	33.3
13	without polymer			

A successful conjugation is indicated by the migration of the protein bands to higher molecular weights in the SDS page analysis according to Figure 3-6. The increased band-width is due to the molecular weight distribution of the HES-, dextran- and PEG- derivatives used and the number of polymer derivatives linked to the protein. In contrast to the hydroxylamino-dextran or hydroxylamino-HES derivatives, the corresponding PEG derivatives migrate into the gel and are also stained, therefore complicating the visualisation of the protein bands.

Short description of the Figures

Figure 1

Figure 1 shows an SDS page analysis of the HES—EPO conjugates, produced according to Example C4.2). For gel electrophoresis a XCell Sure Lock Mini Cell (Invitrogen GmbH, Karlsruhe, D) and a Consort E143 power supply (CONSORTnv, Turnhout, B) were employed. A 10% Bis-Tris gel together with a MOPS SDS running buffer at reducing conditions (both Invitrogen GmbH, Karlsruhe, D) were used according to the manufacture's instruction.

- Lane A: Protein marker SeeBlue®Plus2 (Invitrogen GmbH, Karlsruhe, D) Molecular weight marker from top to bottom: 188 kD, 98 kD, 62 kD, 49 kD, 38 kD, 28 kD, 17 kD, 14 kD, 6 kD, 3 kD
- 5 Lane B: Crude reaction product Example C4.2) (a)
- Lane C: Crude reaction product Example C4.2) (b)
- Lane E: Crude reaction product Example C4.2) (d)
- Lane F: Crude reaction product Example C4.2) (c)
- Lane G: Oxidized EPO according to Example C2).

10

Figure 2

Figure 2 shows an SDS page analysis of the HES—EPO conjugates, produced according to Example C3). For gel electrophoresis a XCell Sure Lock Mini Cell (Invitrogen GmbH, Karlsruhe, D) and a Consort E143 power supply (CONSORTnv, Turnhout, B) were employed. A 10% Bis-Tris gel together with a MOPS SDS running buffer at reducing conditions (both Invitrogen GmbH, Karlsruhe, D) were used according to the manufacture's instruction.

- 20 Lane A: Protein marker Roti-Mark STANDARD (Carl Roth GmbH + Co.KG, Karlsruhe, D) Molecular weight marker from top to bottom: 200 KD, 119 KD, 66 KD, 43 KD, 29 KD, 20 KD, 14.3 KD.
- Lane B: Crude product after conjugation of oxidized hEPO with polymer derivative prepared as described in Example Aa).
- 25 Lane C: Crude product after conjugation of oxidized hEPO with polymer derivative prepared as described in Example Ab).
- Lane D: Crude product after conjugation of oxidized hEPO with polymer derivative prepared as described in Example B).
- Lane E: Reaction control: hEPO without polymer derivative.
- 30 Lane F: Polymer derivative prepared as described in Example Aa).
- Lane G: Polymer derivative prepared as described in Example Ab).

Figures 3-6

Each of Figures 3-6 shows an SDS page analysis of the HES—EPO conjugates, produced according to Example G). For gel electrophoresis a XCell Sure Lock Mini Cell (Invitrogen GmbH, Karlsruhe, D) and a Consort E143 power supply (CONSORTnv, Turnhout, B) were employed. A 10% Bis-Tris gel together with a MOPS SDS running buffer at reducing conditions (both Invitrogen GmbH, Karlsruhe, D) were used according to the manufacture's instruction.

Figure 3

- 10 Lane A: Protein marker Roti-Mark STANDARD (Carl Roth GmbH + Co.KG, Karlsruhe, D)
Molecular weight marker from top to bottom: 200 KD, 119 KD, 66 KD, 43 KD, 29
KD, 20 KD, 14.3 KD.
- Lane B: Crude product after conjugation of oxidized hEPO with polymer derivative A.
- Lane C: Polymer derivative A.
- 15 Lane D: Crude product after conjugation of oxidized hEPO with polymer derivative B.
- Lane E: Polymer derivative B.
- Lane F: Crude product after conjugation of oxidized hEPO with polymer derivative C.
- Lane G: Polymer derivative C.
- Lane H: Crude product after conjugation of oxidized hEPO with polymer derivative D.
- 20 Lane I: Polymer derivative D.
- Lane K: Reaction control: hEPO without polymer derivative.

Figure 4

- 25 Lane A: Protein marker Roti-Mark STANDARD (Carl Roth GmbH + Co.KG, Karlsruhe, D)
Molecular weight marker from top to bottom: 200 KD, 119 KD, 66 KD, 43 KD, 29
KD, 20 KD, 14.3 KD.
- Lane B: Crude product after conjugation of oxidized hEPO with polymer derivative H.
- Lane C: Polymer derivative H.
- 30 Lane D: Crude product after conjugation of oxidized hEPO with polymer derivative I.
- Lane E: Polymer derivative I.
- Lane F: Crude products after conjugation of oxidized hEPO with polymer derivative E.
- Lane G: Polymer derivative E.
- Lane K: Reaction control: hEPO without polymer derivative.

Figure 5

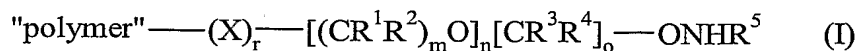
- Lane A: Protein marker Roti-Mark STANDARD (Carl Roth GmbH + Co.KG, Karlsruhe, D)
- 5 Molecular weight marker from top to bottom: 200 KD, 119 KD, 66 KD, 43 KD, 29 KD, 20 KD, 14.3 KD.
- Lane B: Crude product after conjugation of oxidized hEPO with polymer derivative F.
- Lane C: Polymer derivative F.
- Lane D: Reaction control: hEPO without polymer derivative.
- 10 Lane E: Polymer Protein marker Roti-Mark STANDARD (Carl Roth GmbH + Co.KG, Karlsruhe, D) Molecular weight marker from top to bottom: 200 KD, 119 KD, 66 KD, 43 KD, 29 KD, 20 KD, 14.3 KD.
- Lane F: Crude product after conjugation of oxidized hEPO with polymer derivative G.
- Lane G: Polymer derivative G.
- 15 Lane K: Reaction control: hEPO without polymer derivative.

Figure 6

- Lane A: Protein marker Roti-Mark STANDARD (Carl Roth GmbH + Co.KG, Karlsruhe, D)
- 20 Molecular weight marker from top to bottom: 200 KD, 119 KD, 66 KD, 43 KD, 29 KD, 20 KD, 14.3 KD.
- Lane B: Crude product after conjugation of oxidized hEPO with polymer derivative M.
- Lane C: Polymer derivative M.
- Lane D: Crude product after conjugation of oxidized hEPO with polymer derivative J.
- 25 Lane E: Polymer derivative J.
- Lane F: Crude product after conjugation of oxidized hEPO with polymer derivative K.
- Lane G: Polymer derivative K.
- Lane H: Reaction control: hEPO without polymer derivative.
- Lane I: Protein marker Roti-Mark STANDARD (Carl Roth GmbH + Co.KG, Karlsruhe, D)
- 30 Molecular weight marker from top to bottom: 200 KD, 119 KD, 66 KD, 43 KD, 29 KD, 20 KD, 14.3 KD.
- Lane J: Crude product after conjugation of oxidized hEPO with polymer derivative L.
- Lane K: Polymer derivative L.
- Lane L: Reaction control: hEPO without polymer derivative.

Claims

1. Functionalized polymer of formula I



wherein the symbols have the following meanings

"polymer" soluble linear or branched homopolymers or random copolymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, alkylene glycol copolymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids and polysaccharides;

$R^1, R^2, R^3,$

R^4, R^5 hydrogen, alkyl, aryl;

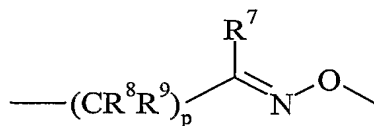
m 2 to 4, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2 ;

n 0 to 20;

o 0 to 20, wherein in the case of $n = 0$, o is not 0, wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4 ;

r 0 or 1;

X $-(CR^8R^9)_pO-$, $-(CR^8R^9)_pS-$, $-(CR^8R^9)_pNR^6-$, $-(CR^8R^9)_pOC(O)-$, $-(CR^8R^9)_pC(O)O-$, $-(CR^8R^9)_pC(G)N(R^{10})O-$, $-(CR^8R^9)_pN(R^{11})O-$,



wherein one or more groups $-(CR^8R^9)-$ may be replaced by W , whereby a chemically reasonable group is formed;

W $O, NR^{12}, C(G)$;

G S, O, NR^{14} ;

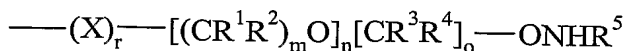
$R^6, R^7, R^8,$

$R^9, R^{10},$

R^{11}, R^{12}, R^{14} hydrogen, alkyl, aryl,

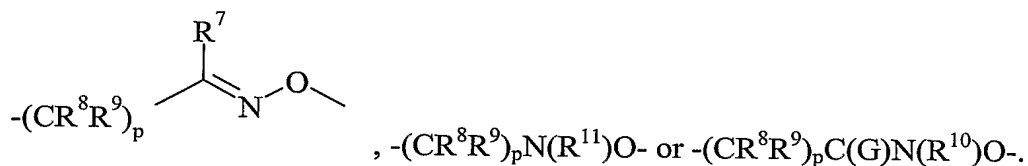
p 0 to 20, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 ;

wherein the group



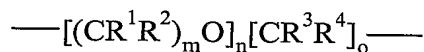
is covalently linked with at least one terminal group or at least one centrally located groups of the "polymer".

2. Functionalized polymer as claimed in claim 1, wherein X is



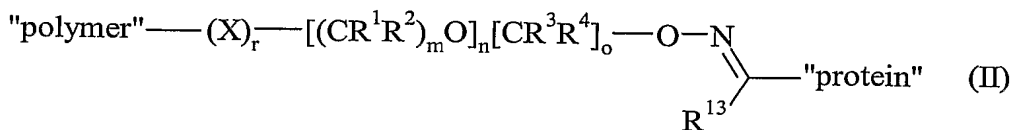
3. Functionalized polymer as claimed in claim 1 or 2, wherein the polymer is hydroxyalkyl starch, dextran or ethylene glycol homopolymer.

4. Functionalized polymer as claimed in any of claims 1 or 3, wherein the group



is $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2-$.

5. Conjugate of formula II



wherein the symbols have the following meanings

"polymer" soluble linear or branched homopolymers or random copolymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, alkylene glycol copolymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane,

ethylene/maleic anhydride copolymer, polyaminoacids and polysaccharides;

$R^1, R^2, R^3,$

R^4 hydrogen, alkyl, aryl;

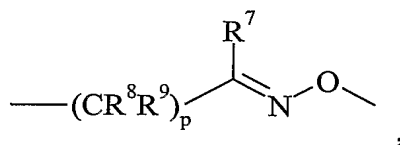
m 2 to 4, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2 ;

n 0 to 20;

o 0 to 20, wherein in the case of $n = 0$, o is not 0, wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4 ;

r 0 or 1;

X $-(CR^8R^9)_pO-$, $-(CR^8R^9)_pS-$, $-(CR^8R^9)_pNR^6-$, $-(CR^8R^9)_pOC(O)-$, $-(CR^8R^9)_pC(O)O-$, $-(CR^8R^9)_pC(G)N(R^{10})O-$, $-(CR^8R^9)_pN(R^{11})O-$,



wherein one or more groups $-(CR^8R^9)-$ may be replaced by W , whereby a chemically reasonable group is formed;

W O , NR^{12} , $C(G)$;

G S , O , NR^{14} ;

$R^6, R^7, R^8,$

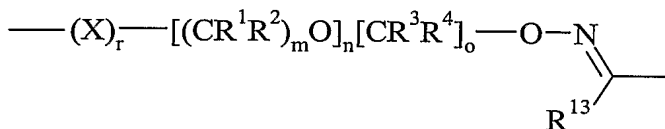
$R^9, R^{10},$

R^{11}, R^{12}, R^{14} hydrogen, alkyl, aryl, preferably hydrogen,

p 0 to 20, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 ;

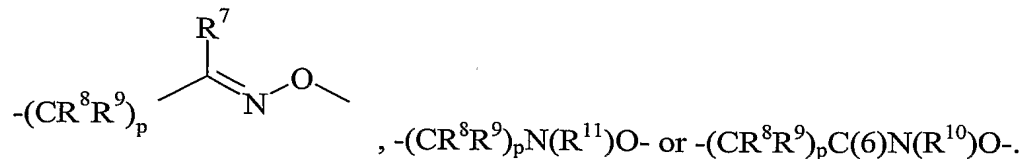
R^{13} hydrogen, alkyl, aryl;

“protein” amino acid sequence prepared by reaction of at least 2 amino acids wherein the group



is covalently linked with at least one terminal group or at least one centrally located group of the “polymer” and the “protein”.

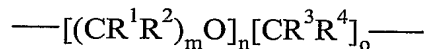
6. Conjugate as claimed in claim 5, wherein X is



7. Conjugate as claimed in claim 5 or 6, wherein the polymer is hydroxyalkyl starch, dextran or ethylene glycol homopolymer.

8. Conjugate as claimed in any of claims 5 to 7, wherein the "protein" is selected from the group consisting of EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII and APC.

9. Conjugate as claimed in any of claims 5 or 8, wherein the group

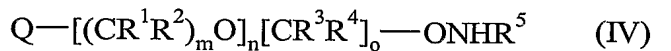


is $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2-$.

10. Process for preparing a functionalized polymer comprising the step of reacting a polymer of formula III



with a compound of formula IV



wherein the symbols have the following meanings

"polymer" soluble linear or branched homopolymers or random copolymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, alkylene glycol copolymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids and polysaccharides;

$R^1, R^2, R^3,$

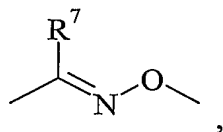
R^4, R^5 hydrogen, alkyl, aryl;

m 2 to 4, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2 ;

n 0 to 20;

o 0 to 20, wherein in the case of $n = 0$, o is not 0, wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4 ;

Y and Q functional groups, which are suitable to react together to give one of the following linking groups -O-, -S-, -NR⁶-, -OC(O)-, -C(O)O-, -C(G)N(R¹⁰)O-, -N(R¹¹)O-,



wherein one or more groups $-(CR^8R^9)-$ may be replaced by W, whereby a chemically reasonable group is formed;

W O, NR¹², C(G);

G S, O, NR¹⁴;

$R^6, R^7, R^8,$

$R^9, R^{10},$

R^{11}, R^{12}, R^{14} hydrogen, alkyl, aryl;

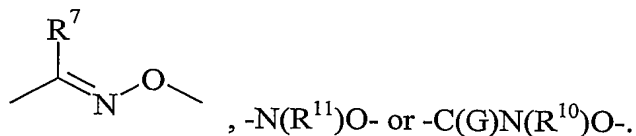
p 0 to 20, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 ;

wherein the group

$-(CR^8R^9)_p-Y$

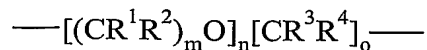
is covalently linked with terminal groups or centrally located groups of the "polymer".

11. Process as claimed in claim 10, wherein Y and Q are functional groups, which are suitable to react together to give the following linking group



12. Process as claimed in claim 10 or 11, wherein the polymer is hydroxyalkyl starch, dextran or ethylene glycol homopolymer.

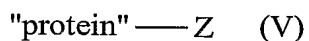
13. Process as claimed in any of claims 10 to 12, wherein the group



is $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2-$.

14. Functionalized polymer as obtainable by a process as claimed in any of claims 10 to 13.

15. Process for preparing a conjugate, comprising the step of reacting a functionalized polymer as claimed in any of claims 1 to 4 or 14 with a functionalized protein of formula V



wherein Z is a group comprising a carbonyl group or a group which is suitable of forming a carbonyl group or another group which is reactable with the functionalized polymer, wherein Z is covalently linked with least one terminal group or least one centrally located group of the "protein".

16. Process as claimed in claim 15, wherein a functionalized polymer as claimed in any of claims 2 to 4 is employed.

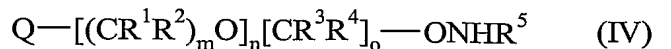
17. Process as claimed in claim 15 or 16, wherein the "protein" is selected from the group consisting of EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII and APC.

18. Process for preparing a conjugate, comprising the steps

a) reacting a polymer of formula III



with a compound of formula IV



wherein the symbols have the following meanings

“polymer” soluble linear or branched homopolymers or random copolymers and derivatives thereof selected from the group consisting of alkylene glycol homopolymers, alkylene glycol copolymers, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids and polysaccharides;

$R^1, R^2, R^3,$

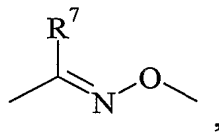
R^4, R^5 hydrogen, alkyl, aryl;

m 2 to 4, wherein the residues R^1 and R^2 may be the same or different in the m groups CR^1R^2 ;

n 0 to 20;

o 0 to 20, wherein in the case of $n = 0$, o is not 0, wherein the residues R^3 and R^4 may be the same or different in the o groups CR^3R^4 ;

Y and Q functional groups, which are suitable to react together to give one of the following linking groups $-O-$, $-S-$, $-NR^6-$, $-OC(O)-$, $-C(O)O-$, $-C(G)N(R^{10})O-$, $-N(R^{11})O-$,



wherein one or more groups $-(CR^8R^9)-$ may be replaced by W , whereby a chemically reasonable group is formed;

W $O, NR^{12}, C(G)$;

G S, O, NR^{14} ;

$R^6, R^7, R^8,$

$R^9, R^{10},$

R^{11}, R^{12}, R^{14} hydrogen, alkyl, aryl;

p 0 to 20, wherein the residues R^8 and R^9 may be the same or different in the p groups CR^8R^9 ;

wherein the group

$-(CR^8R^9)_p-Y$

is covalently linked with least one terminal group and/or least one centrally located group of the "polymer", wherein a functionalized polymer is obtained, and

b) reacting the functionalized polymer obtained in step a) with a functionalized protein of formula V

"protein" — Z (V)

wherein Z is a group comprising a carbonyl group or a group which is suitable of forming a carbonyl group or another group which is reactable with the functionalized polymer, wherein Z is covalently linked with least one terminal group and/or least one centrally located group of the "protein".

19. Conjugate as obtainable by a process as claimed in any of claims 15 to 18.

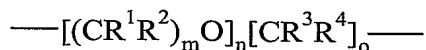
20. A conjugate as claimed in any of claims 5 to 9 or 19 for use in a method for the treatment of the human or animal body.

21. A pharmaceutical composition comprising in a therapeutically effective amount a conjugate as claimed in any of claims 5 to 9 or 19.

22. The pharmaceutical composition as claimed in claim 21, further comprising at least one pharmaceutically acceptable diluent, adjuvant, or carrier.

23. Functionalized polymer as claimed in any of claims 1 to 3, wherein o is from 2 to 20.

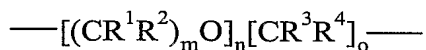
24. Functionalized polymer as claimed in any of claims 1 to 3, wherein the group



is $\text{---CH}_2\text{CH}_2(\text{CH}_3)\text{CH}_2\text{CH}_2\text{---}$.

25. Conjugate as claimed in any of claims 5 to 8, wherein o is from 2 to 20.

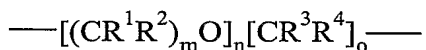
26. Conjugate as claimed in any of claims 5 to 8, wherein the group



is $\text{---CH}_2\text{CH}_2(\text{CH}_3)\text{CH}_2\text{CH}_2\text{---}$.

27. The process as claimed in any of claims 10 to 12, wherein o is from 2 to 20.

28. The process as claimed in any of claims 10 to 12, wherein the group



is $\text{---CH}_2\text{CH}_2(\text{CH}_3)\text{CH}_2\text{CH}_2\text{---}$.

29. Functionalized polymer as obtainable by a process as claimed in claim 27 or 28.

30. The process as claimed in claim 15 or 17, wherein a functionalized polymer as claimed in any of claims 23, 24 or 29 is reacted with the functionalized protein of formula V.

31. The process as claimed in claim 18, wherein o is from 2 to 20.

32. Conjugate obtainable by a process as claimed in claim 30.

33. A conjugate as claimed in any of claims 25, 26 or 32 for use in a method for the treatment of the human or animal body.

34. A pharmaceutical composition comprising in a therapeutically effective amount a conjugate as claimed in any of claims 25, 26 or 32.

35. The pharmaceutical composition as claimed in claim 34, further comprising at least one pharmaceutically acceptable diluent, adjuvant, or carrier.

Fig. 1

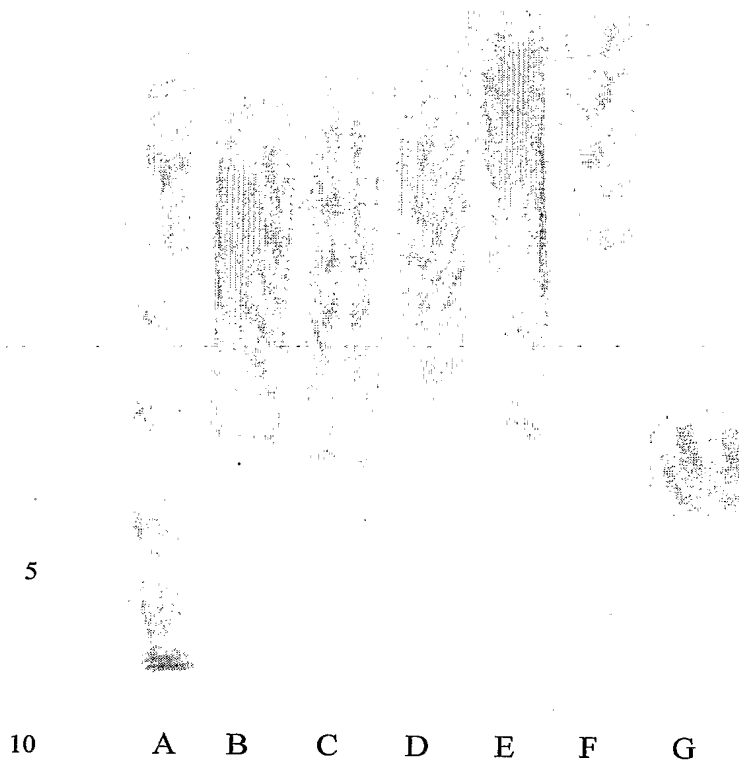
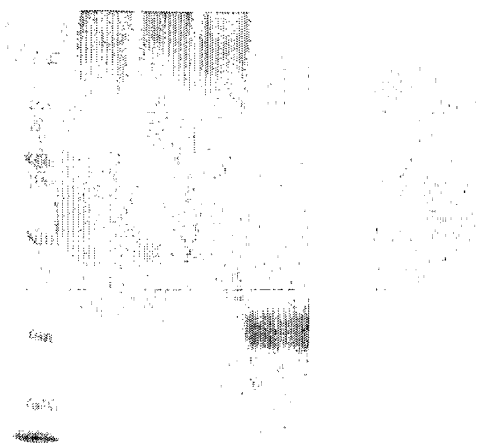


Fig. 2



5 Lane

A B C D E F G

Fig. 3

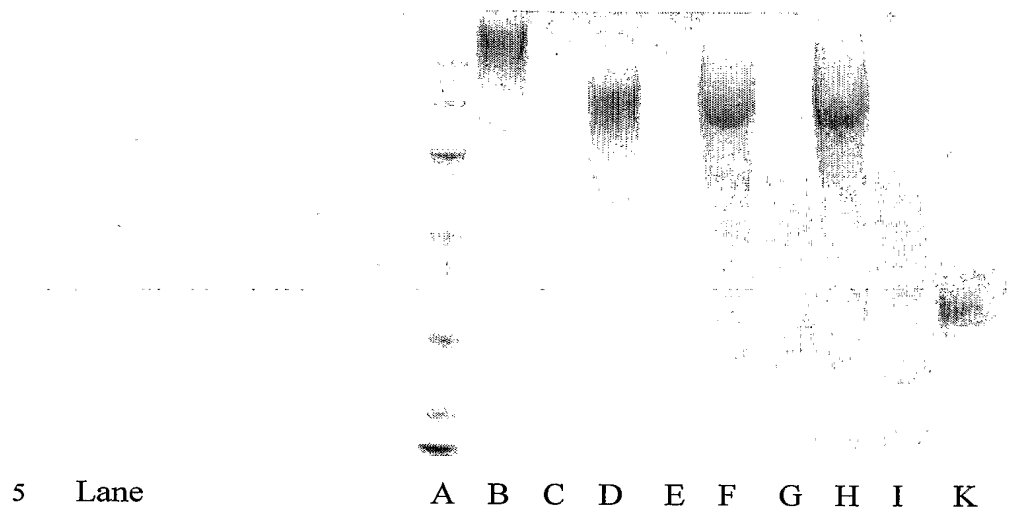


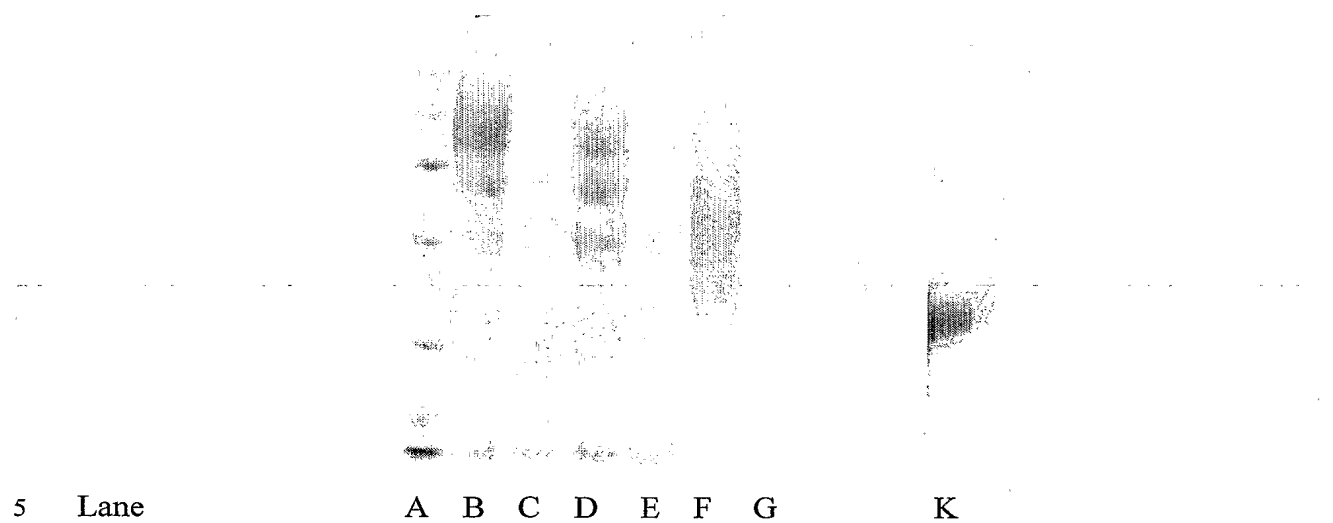
Fig. 4

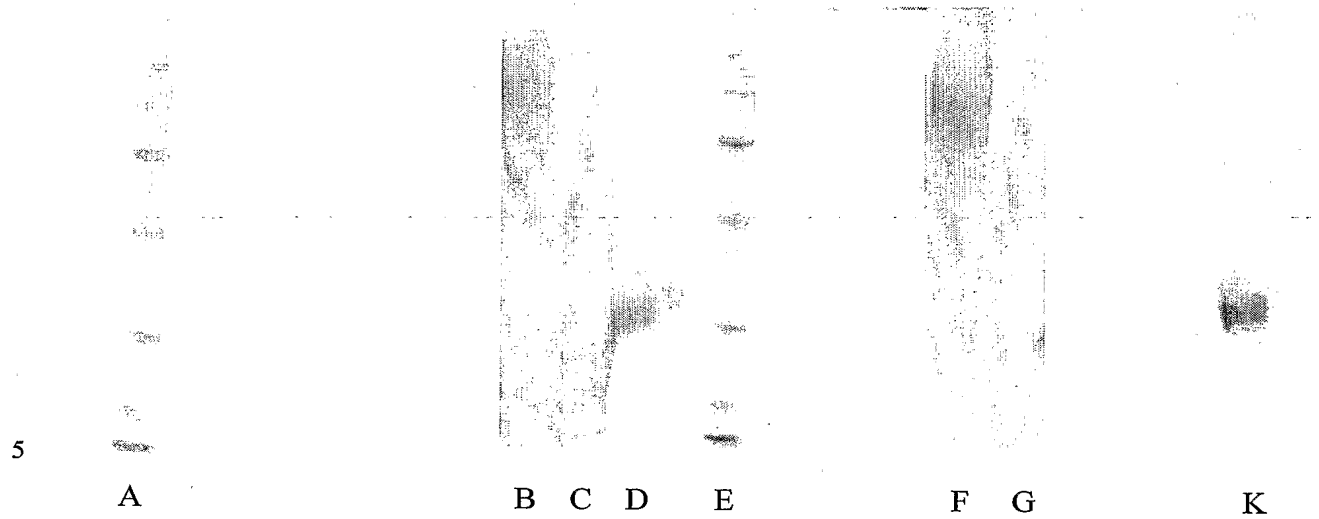
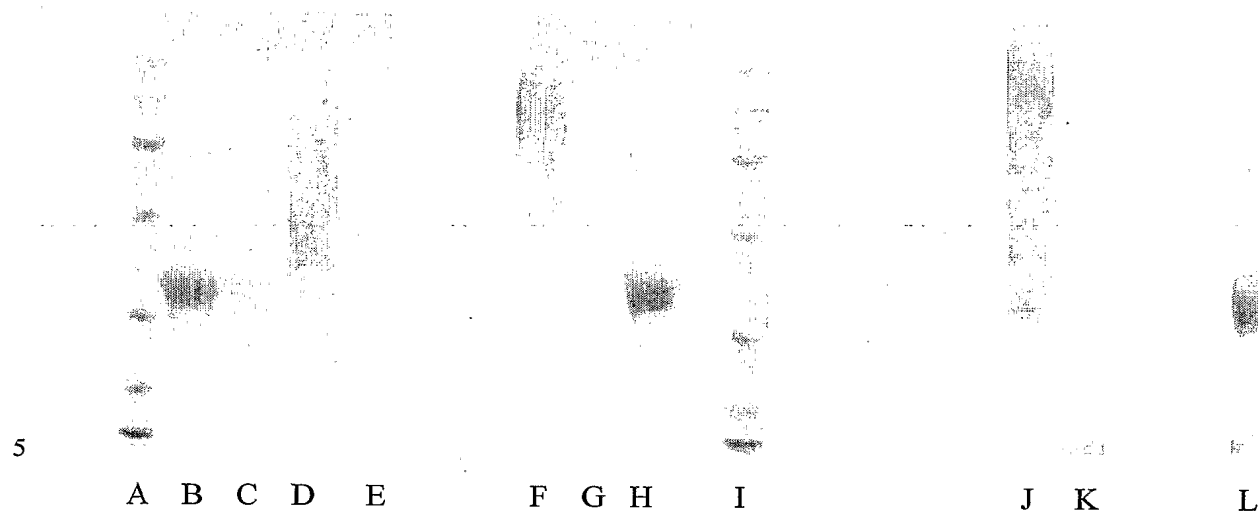
Fig. 5

Fig. 6

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04005872.9 11 March 2004 (11.03.2004) EP
60/552,157 11 March 2004 (11.03.2004) US

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(74) Agent: **WICHMANN, Hendrik**; Isenbruck Bösl Hörschler Wichmann Huhn, Prinzregentenstrasse 68, 81675 München (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations
- of inventorship (Rule 4.17(iv)) for US only
- of inventorship (Rule 4.17(iv)) for US only

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CONJUGATES OF A POLYMER AND A PROTEIN LINKED BY AN OXIME LINKING GROUP

(57) Abstract: The present invention relates to polymers functionalized by an aminooxy group or a derivative thereof, conjugates, wherein the functionalized polymers are covalently coupled with a protein by an oxime linking group, a process for preparing the functionalized polymers, a process for preparing the conjugates, functionalized polymers as obtainable by the process of the present invention, conjugates as obtainable by the process of the present invention, and pharmaceutical compositions comprising at least one conjugate of the present invention and the use of said conjugates and compositions for the prophylaxis or therapy of the human or animal body.



WO 2005/014024 A3

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	A61K38/18	A61K38/19	C07K14/505	C07K14/52	C07K1/107
	A61K47/48	C07K2/00	C08B31/00	C08B37/00	
According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED					
Minimum documentation searched (classification system followed by classification symbols)					
IPC 7	A61K	C07K	C08B		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
EPO-Internal, BIOSIS, EMBASE, MEDLINE, COMPENDEX, CHEM ABS Data					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category *	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	EP 0 605 963 A (ORTHO PHARMA CORP) 13 July 1994 (1994-07-13) abstract; claims 4,6 page 7, line 45 - line 58 page 15 - page 17 page 19				1-23,25, 27,29-35
X	----- WO 96/41813 A (OFFORD ROBIN E ; GAERTNER HUBERT F (FR)) 27 December 1996 (1996-12-27) abstract; claims 5-7 page 11, line 3 - line 10 page 15, line 23 page 19, line 21 - line 22 page 23, line 3 - line 7 page 23, line 24 - page 24, line 23 ----- -/--				1-35
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.					
* Special categories of cited documents : *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *& document member of the same patent family					
Date of the actual completion of the international search			Date of mailing of the international search report		
25 January 2005			18.03.05		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016			Authorized officer Jenn, T		

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 2003/191291 A1 (CRESSMAN SONYA ET AL) 9 October 2003 (2003-10-09) figures 14A,14B; example 2 -----	1-35
A	GAERTNER H F ET AL: "SITE-SPECIFIC ATTACHMENT OF FUNCTIONALIZED POLY(ETHYLENE GLYCOL) TO THE AMINO TERMINUS OF PROTEINS" BIOCONJUGATE CHEMISTRY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, US, vol. 7, no. 1, January 1996 (1996-01), pages 38-44, XP000646874 ISSN: 1043-1802 cited in the application * Scheme 1 * abstract; figures 4-7 -----	1-23, 25, 27, 29-35
X	VILASECA L ET AL: "PROTEIN CONJUGATES OF DEFINED STRUCTURE: SYNTHESIS AND USE OF A NEW CARRIER MOLECULE" BIOCONJUGATE CHEMISTRY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, US, vol. 4, no. 6, 1 November 1993 (1993-11-01), pages 515-520, XP000417288 ISSN: 1043-1802 abstract; figures 1,3 page 516, right-hand column, paragraph 4 -----	1-35
A	ROSE KEITH: "Facile synthesis of homogeneous artificial proteins" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 116, no. 1, 12 January 1994 (1994-01-12), pages 30-33, XP002301786 ISSN: 0002-7863 figure 1 page 30, right-hand column, paragraph 2 page 31, left-hand column, paragraph 1 page 33, right-hand column, paragraph 2 -----	1-35
P,A	WO 2004/009082 A (GEN HOSPITAL CORP ; PAPISOV MIKHAIL I (US); YURKOVETSKIY ALEXANDER (US) 29 January 2004 (2004-01-29) claims 14,28,29 page 21, paragraph 21 - paragraph 22 page 53, paragraph 14 - page 54, paragraph 150 page 80; compound 10 page 96, paragraph 297 - paragraph 298 ----- -/--	1-35

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	<p>JONES DAVID S ET AL: "Multivalent poly(ethylene glycol)-containing conjugates for in vivo antibody suppression." BIOCONJUGATE CHEMISTRY, vol. 14, no. 6, November 2003 (2003-11), pages 1067-1076, XP002302390 ISSN: 1043-1802 abstract; figure 1 * Schemes 1-8 * page 1072, left-hand column, paragraph 3 - right-hand column, paragraph 4 page 1074, left-hand column, line 1 - line 3</p>	1-35
P,X	<p>----- SADAMOTO R ET AL: "CONTROL OF BACTERIA ADHESION BY CELL-WALL ENGINEERING" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 126, 31 March 2004 (2004-03-31), pages 3755-3761, XP008035406 ISSN: 0002-7863 * Scheme 1 *</p>	1-23,25, 27,29-35
P,X	<p>----- EP 1 398 328 A (FRESENIUS KABI DE GMBH) 17 March 2004 (2004-03-17) abstract; claim 66 page 33, paragraph 295 - paragraph 296 page 34, paragraph 301 - paragraph 302</p>	1-23,25, 27,29-35
A	<p>----- JONES D S ET AL: "A convenient synthesis of N-(tert-butyloxycarbonyl)aminoxy ethers" TETRAHEDRON LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 41, no. 10, 4 March 2000 (2000-03-04), pages 1531-1533, XP004189789 ISSN: 0040-4039 abstract; table 1</p>	1-23,25, 27,29-35
A	<p>----- ROHRLING J ET AL: "Synthesis and testing of a novel fluorescence label for carbonyls in carbohydrates and cellulosics" SYNLETT 2001 GERMANY, no. 5, 2001, pages 682-684, XP001203540 ISSN: 0936-5214 * Scheme 1 *</p> <p>----- -/--</p>	1-23,25, 27,29-35

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/080979 A (SOMMERMEYER KLAUS ; FRIE SVEN (DE); EICHNER WOLFRAM (DE); SCHARPF ROLA) 17 October 2002 (2002-10-17) abstract; claims 1,5,63,64 page 29, line 10 - page 30, line 6 -----	1-35
A	WO 95/06058 A (ROYAL FREE HOSP SCHOOL MED ; FRANCIS GILLIAN ELIZABETH (GB); FISHER DE) 2 March 1995 (1995-03-02) claims 15,16,18 page 33, line 7 - line 9 page 37, line 22 -----	1-35
A	WO 96/40662 A (EMERALD DIAGNOSTICS INC ; CELLPRO INC (US)) 19 December 1996 (1996-12-19) claims 4,6 -----	1-35
A	NEDOSPASOV A A ET AL: "SYNTHESIS AND SOME PROPERTIES OF AMINOOXYALKYLCELLULOSES" BULLETIN OF THE ACADEMY OF SCIENCES OF THE USSR. DIVISION OF CHEMICAL SCIENCE, CONSULTANTS BUREAU. NEW YORK, US, vol. 25, 1976, pages 1105-1110, XP002025380 page 1105, paragraph 3 page 1106 -----	1-35
A	HAMMA TOMOKO ET AL: "4-(2-aminooxyethoxy)-2-(ethylureido)quino line-oligonucleotide conjugates: synthesis, binding interactions, and derivatization with peptides." BIOCONJUGATE CHEMISTRY. 2003 MAR-APR, vol. 14, no. 2, March 2003 (2003-03), pages 320-330, XP002301933 ISSN: 1043-1802 abstract; figures 1,4 * Scheme 2 * -----	1-35
A	GB 1 419 080 A (AKTIESELSKABET CHEMINOVA) 24 December 1975 (1975-12-24) abstract; claims 1-36 table 1; compounds 8,17-19 -----	1-35
A	SAWAIKAR, D. D. ET AL: "Products active on mosquitoes. Part VII. Synthesis and biological activity of longifolene derivatives" INDIAN JOURNAL OF CHEMISTRY, SECTION B: ORGANIC CHEMISTRY INCLUDING MEDICINAL CHEMISTRY, 34B(9), 832-5 CODEN: IJSBDB; ISSN: 0376-4699, 1995, XP9043047 abstract; figure 1 -----	1-35
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WEBB R R 2ND ET AL: "Synthesis of 1-(aminooxy)-4-(3-nitro-2-pyridyl)dithio! butane and 1-(aminooxy)-4-(3-nitro-2-pyridyl)dithio! but-2-ene, novel heterobifunctional cross-linking reagents." BIOCONJUGATE CHEMISTRY, vol. 1, no. 2, March 1990 (1990-O3), pages 96-99, XP002223790 ISSN: 1043-1802 abstract Chart I, Scheme I, Scheme III page 96, left-hand column, paragraph 1 page 96, right-hand column, paragraph 1</p>	1-35
A	<p>MIKOLA H ET AL: "Introduction of aliphatic amino and hydroxy groups to keto steroids using O-substituted hydroxylamines." BIOCONJUGATE CHEMISTRY. 1992 MAR-APR, vol. 3, no. 2, March 1992 (1992-O3), pages 182-186, XP000262175 ISSN: 1043-1802 Schemes I-IV abstract page 182, left-hand column, paragraph 3 page 184, left-hand column, paragraph 8 - page 185, left-hand column, paragraph 1</p>	1-35
A	<p>EP 0 243 929 A2 (F. HOFFMANN-LA ROCHE & CO. AKTIENGESELLSCHAFT; OFFORD, ROBIN EWART; RO) 4 November 1987 (1987-11-04) abstract; claims 11,28; examples 16-19 page 11, line 32 - line 37</p>	1-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/008820

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
1-35 (all in parts, concerning invention 1 and invention 5)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Inventions 1-4: claims 1-23, 25, 27, 29-35, all in part

A functionalized polymer of formula (I) according to claim 1, wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}_n(\text{CR}_3\text{R}_4)_o-$ is $\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$, and R_5 is H;

A process according to claim 10 for preparing a functionalized polymer of formula (I), wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}_n(\text{CR}_3\text{R}_4)_o-$ is $\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$, and R_5 is H; A functionalized polymer obtained by said process wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}_n(\text{CR}_3\text{R}_4)_o-$ is $\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$, and R_5 is H; A conjugate of formula (II) according to claim 5, wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}_n(\text{CR}_3\text{R}_4)_o-$ is $\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$, and "protein" is EP0;

A process according to claim 15, for preparing a conjugate of formula (II), wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}_n(\text{CR}_3\text{R}_4)_o-$ is $\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$, and "protein" is EP0.

A process according to claim 18, for preparing a conjugate of formula (II), wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}_n(\text{CR}_3\text{R}_4)_o-$ is $\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2$, and "protein" is EP0.

A conjugate obtained by said process.

Said conjugate for use in a method of treatment;

A pharmaceutical composition comprising said conjugate.

and wherein for said polymer, conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch (Invention 1), or dextran (Invention 2), or an ethylene glycol homopolymer (Invention 3), or is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer (Invention 4).

Inventions 5-8: claims 1-3, 5-8, 10-12, 14-35, all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A functionalized polymer of formula (I) according to claim 1, wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}-(\text{CR}_3\text{R}_4)_n-$ is $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2$, and R_5 is H;

A process according to claim 10 for preparing a functionalized polymer of formula (I), wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}-(\text{CR}_3\text{R}_4)_n-$ is $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2$, and R_5 is H;

A functionalized polymer obtained by said process wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}-(\text{CR}_3\text{R}_4)_n-$ is $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2$, and R_5 is H;

A conjugate of formula (II) according to claim 5, wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}-(\text{CR}_3\text{R}_4)_n-$ is $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2$, and "protein" is EPO;

A process according to claim 15, for preparing a conjugate of formula (II), wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}-(\text{CR}_3\text{R}_4)_n-$ is $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2$, and "protein" is EPO.

A process according to claim 18, for preparing a conjugate of formula (II), wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}-(\text{CR}_3\text{R}_4)_n-$ is $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2$, and "protein" is EPO.

A conjugate obtained by said process.

Said conjugate for use in a method of treatment;

A pharmaceutical composition comprising said conjugate.

and wherein for said polymer, conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch (Invention 5), or dextran (Inv. 6), or an ethylene glycol homopolymer (Inv. 7), or is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer (Inv. 8).

Inventions 9-12: claims 1-3, 5-8, 10-12, 14-23, 25, 27, 29-35, all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A functionalized polymer of formula (I) according to claim 1, wherein o is from 2 to 20, and R5 is H, excluding such a polymer wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is either $-(CH_2)_{20}(CH_2)_2-$ or $-(CH_2)_2CH(CH_3)(CH_2)_2-$;

A process according to claim 10 for preparing a functionalized polymer of formula (I), wherein o is from 2 to 20, and R5 is H, excluding the preparation of such a polymer wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is either $-(CH_2)_{20}(CH_2)_2-$ or $-(CH_2)_2CH(CH_3)(CH_2)_2-$;

A functionalized polymer obtained by said process wherein o is from 2 to 20, and R5 is H, excluding such a polymer wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is either $-(CH_2)_{20}(CH_2)_2-$ or $-(CH_2)_2CH(CH_3)(CH_2)_2-$;

A conjugate of formula (II) according to claim 5, wherein o is from 2 to 20, and "protein" is EP0, excluding such a conjugate wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is either $-(CH_2)_{20}(CH_2)_2-$ or $-(CH_2)_2CH(CH_3)(CH_2)_2-$;

A process according to claim 15, for preparing a conjugate of formula (II), wherein o is from 2 to 20, and "protein" is EP0, excluding the preparation of such a conjugate wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is either $-(CH_2)_{20}(CH_2)_2-$ or $-(CH_2)_2CH(CH_3)(CH_2)_2-$;

A process according to claim 18, for preparing a conjugate of formula (II), wherein o is from 2 to 20, and "protein" is EP0, excluding the preparation of such a conjugate wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is either $-(CH_2)_{20}(CH_2)_2-$ or $-(CH_2)_2CH(CH_3)(CH_2)_2-$;

A conjugate obtained by said process.

Said conjugate for use in a method of treatment;

A pharmaceutical composition comprising said conjugate.

and wherein for said polymer, conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch (Invention 9), or dextran (Inv. 10). or

an ethylene glycol homopolymer (Inv. 11), or

is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer (Inv. 12).

Inventions 13-16: claims 1-3, 5-23, 25, 27, 29-35, all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A functionalized polymer of formula (I) according to claim 1, wherein o is 0 or 1, and R5 is H;
A process according to claim 10 for preparing a functionalized polymer of formula (I), wherein o is 0 or 1, and R5 is H;
A functionalized polymer obtained by said process wherein o is 0 or 1, and R5 is H;
A conjugate of formula (II) according to claim 5, wherein o is 0 or 1, and "protein" is EP0;
A process according to claim 15, for preparing a conjugate of formula (II), wherein o is 0 or 1, and "protein" is EP0.
A process according to claim 18, for preparing a conjugate of formula (II), wherein o is 0 or 1, and "protein" is EP0.
A conjugate obtained by said process.
Said conjugate for use in a method of treatment;
A pharmaceutical composition comprising said conjugate.

and wherein for said polymer, conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch (Invention 13), or dextran (Inv. 14), or an ethylene glycol homopolymer (Inv. 15), or is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer (Inv. 16).

Inventions 17-52: claims 5-9 and 15-22, all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A conjugate of formula (II) according to claim 5, wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is $CH_2CH_2OCH_2CH_2$,
 A process according to claim 15, for preparing a conjugate of formula (II), wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is $CH_2CH_2OCH_2CH_2$,
 A process according to claim 18, for preparing a conjugate of formula (II), wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is $CH_2CH_2OCH_2CH_2$,
 A conjugate obtained by said process,
 Said conjugate for use in a method of treatment,
 A pharmaceutical composition comprising said conjugate,

and wherein for said conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch, and the "protein" is G-CSF (Invention 17), or Factor VII (Inv. 18), or Factor IX (Inv. 19), or IFN beta (Inv. 20), or AT III (Inv. 21), or A1AT (Inv. 22), or Factor VIII (Inv. 23), or APC (Inv. 24), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 25), or the "polymer" is dextran, and the "protein" is G-CSF (Inv. 26), or Factor VII (Inv. 27), or Factor IX (Inv. 28), or FN beta (Inv. 29), or AT III (Inv. 30), or A1AT (Inv. 31), or Factor VIII (Inv. 32), or APC (Inv. 33), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 34), or the "polymer" is an ethylene glycol homopolymer and the "protein" is G-CSF (Inv. 35), or Factor VII (Inv. 36), or Factor IX (Inv. 37), or IFN beta (Inv. 38), or AT III (Inv. 39), or A1AT (Inv. 40), or Factor VIII (Inv. 41), or APC (Inv. 42), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 43), or the "polymer" is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer and the "protein" is G-CSF (Inv. 44), or Factor VII (Inv. 45), or Factor IX (Inv. 46), or IFN beta (Inv. 47), or AT III (Inv. 48), or A1AT (Inv. 49), or Factor VIII (Inv. 50), or APC (Inv. 51), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 52).

Inventions 53-88: claims 5-9 and 15-22, all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A conjugate of formula (II) according to claim 5, wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is $CH_2CH_2CH(CH_3)CH_2CH_2$,
 A process according to claim 15, for preparing a conjugate of formula (II), wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is $CH_2CH_2CH(CH_3)CH_2CH_2$,
 A process according to claim 18, for preparing a conjugate of formula (II), wherein the group $-(CR_1R_2)mO!n'CR_3R_4!o-$ is $CH_2CH_2CH(CH_3)CH_2CH_2$,
 A conjugate obtained by said process.
 Said conjugate for use in a method of treatment;
 A pharmaceutical composition comprising said conjugate.

and wherein for said conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch, and the "protein" is G-CSF (Invention 53), or
 Factor VII (Inv. 54), or Factor IX (Inv. 55), or IFN beta (Inv. 56), or AT III (Inv. 57), or A1AT (Inv. 58), or Factor VIII (Inv. 59), or APC (Inv. 60), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 61), or
 the "polymer" is dextran, and the "protein" is G-CSF (Invention 62), or
 Factor VII (Inv. 63), or Factor IX (Inv. 64), or IFN beta (Inv. 65), or AT III (Inv. 66), or A1AT (Inv. 67), or Factor VIII (Inv. 68), or APC (Inv. 69), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 70), or
 the "polymer" is an ethylene glycol homopolymer and the "protein" is G-CSF (Invention 71), or
 Factor VII (Inv. 72), or Factor IX (Inv. 73), or IFN beta (Inv. 74), or AT III (Inv. 75), or A1AT (Inv. 76), or Factor VIII (Inv. 77), or APC (Inv. 78), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 79), or
 the "polymer" is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer and the "protein" is G-CSF (Invention 80), or
 Factor VII (Inv. 81), or Factor IX (Inv. 82), or IFN beta (Inv. 83), or AT III (Inv. 84), or A1AT (Inv. 85), or Factor VIII (Inv. 86), or APC (Inv. 87), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 88).

 Inventions 89-124: claims 1-3, 5-8, 10-12, 14-23, 25, 27, 29-35,
 all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A conjugate of formula (II) according to claim 5, wherein o is from 2 to 20, excluding such a conjugate wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}!n'(\text{CR}_3\text{R}_4)_o-$ is either $-(\text{CH}_2)_{20}(\text{CH}_2)_2-$ or $-(\text{CH}_2)_2\text{CH}(\text{CH}_3)(\text{CH}_2)_2-$;

A process according to claim 15, for preparing a conjugate of formula (II), wherein o is from 2 to 20, excluding the preparation of such a conjugate wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}!n'(\text{CR}_3\text{R}_4)_o-$ is either $-(\text{CH}_2)_{20}(\text{CH}_2)_2-$ or $-(\text{CH}_2)_2\text{CH}(\text{CH}_3)(\text{CH}_2)_2-$;

A process according to claim 18, for preparing a conjugate of formula (II), wherein o is from 2 to 20, excluding the preparation of such a conjugate wherein the group $-(\text{CR}_1\text{R}_2)_m\text{O}!n'(\text{CR}_3\text{R}_4)_o-$ is either $-(\text{CH}_2)_{20}(\text{CH}_2)_2-$ or $-(\text{CH}_2)_2\text{CH}(\text{CH}_3)(\text{CH}_2)_2-$;

A conjugate obtained by said process.

Said conjugate for use in a method of treatment;

A pharmaceutical composition comprising said conjugate.

and wherein for said conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch, and the "protein" is G-CSF (Invention 89), or

Factor VII, or Factor IX, or IFN beta, or AT III, or A1AT, or Factor VIII, or APC, or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 90-97), or

the "polymer" is dextran, and the "protein" is G-CSF (Inv. 98), or

Factor VII, or Factor IX, or IFN beta, or AT III, or A1AT, or Factor VIII, or APC, or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 99-106), or

the "polymer" is an ethylene glycol homopolymer and the "protein" is G-CSF (Inv. 107), or

Factor VII, or Factor IX, or IFN beta, or AT III, or A1AT, or Factor VIII, or APC, or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 108-115), or

the "polymer" is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer and the "protein" is G-CSF (Inv. 116), or

Factor VII, or Factor IX, or IFN beta, or AT III, or A1AT, or Factor VIII, or APC, or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 117-124).

Inventions 125-160: claims 1-3, 5-23, 25, 27, 29-35, all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A conjugate of formula (II) according to claim 5, wherein o is 0 or 1;

A process according to claim 15, for preparing a conjugate of formula (II), wherein o is 0 or 1;

A process according to claim 18, for preparing a conjugate of formula (II), wherein o is 0 or 1;

A conjugate obtained by said process.

Said conjugate for use in a method of treatment;

A pharmaceutical composition comprising said conjugate.

and wherein for said conjugate, process and/or composition, the "polymer" is hydroxyalkyl starch, and the "protein" is G-CSF (Inv. 125), or

Factor VII (Inv. 126), or Factor IX (Inv. 127), or IFN beta (Inv. 128), or AT III (Inv. 129), or A1AT (Inv. 130), or Factor VIII (Inv. 131), or APC (Inv. 132), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 133), or the "polymer" is dextran, and the "protein" is G-CSF (Inv. 134), or

Factor VII (Inv. 135), or Factor IX (Inv. 136), or IFN beta (Inv. 137), or AT III (Inv. 138), or A1AT (Inv. 139), or Factor VIII (Inv. 140), or APC (Inv. 141), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 142), or the "polymer" is an ethylene glycol homopolymer and the "protein" is G-CSF (Inv. 143), or

Factor VII (Inv. 144), or Factor IX (Inv. 145), or IFN beta (Inv. 146), or AT III (Inv. 147), or A1AT (Inv. 148), or Factor VIII (Inv. 149), or APC (Inv. 150), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 151), or the "polymer" is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer and the "protein" is G-CSF (Inv. 152), or

Factor VII (Inv. 153), or Factor IX (Inv. 154), or IFN beta (Inv. 155), or AT III (Inv. 156), or A1AT (Inv. 157), or Factor VIII (Inv. 158), or APC (Inv. 159), or an amino acid sequence prepared by reaction of at least two amino acids, and different from EPO, G-CSF, Factor VII, Factor IX, IFN beta, AT III, A1AT, Factor VIII or APC (Inv. 160).

Inventions 161-164: claims 1-4, 10-14, 23, 24 and 27-29 all in part

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A functionalized polymer of formula (I) according to claim 1, wherein R5 is alkyl or aryl;

A process according to claim 10 for preparing a functionalized polymer of formula (I), wherein R5 is alkyl or aryl;

A functionalized polymer obtained by said process wherein R5 is alkyl or aryl;

and wherein the "polymer" is hydroxyalkyl starch (Invention 161), or dextran (Inv. 162), or an ethylene glycol homopolymer (Inv. 163), or is different from hydroxyalkyl starch, dextran and ethylene glycol homopolymer (Inv. 164).

Invention 165: claims 10-14 and 27-29, all in part

A process as defined in claim 10, for preparing a functionalized polymer different from a functionalized polymer of formula (I) according to claim 1,
Said functionalized polymer different from a functionalized polymer of formula (I) according to claim 1.

Invention 166: claims 15-22, 25, 26 and 30-35, all in part

A process as defined in claim 15, for preparing a conjugate different from a conjugate of formula (II) according to claim 5,

A process as defined in claim 18, for preparing a conjugate different from a conjugate of formula (II) according to claim 5,

A conjugate obtained by said process,
Said conjugate for use in a method of treatment,
A pharmaceutical composition comprising said conjugate.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2004/008820

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0605963	A	13-07-1994	AU 5238393 A	23-06-1994
			EP 0605963 A2	13-07-1994
			FI 935485 A	10-06-1994
			JP 7196925 A	01-08-1995
			KR 254650 B1	01-05-2000
			KR 257643 B1	15-05-2000
			NO 934477 A	10-06-1994
			NZ 250375 A	26-07-1995
			CA 2110543 A1	10-06-1994
			ZA 9309214 A	08-06-1995
WO 9641813	A	27-12-1996	AU 7327296 A	09-01-1997
			CA 2204726 A1	27-12-1996
			EP 0788375 A2	13-08-1997
			WO 9641813 A2	27-12-1996
			JP 10509208 T	08-09-1998
US 2003191291	A1	09-10-2003	AU 7890501 A	22-03-2002
			BG 107590 A	31-12-2003
			BR 0113623 A	22-06-2004
			CA 2412277 A1	14-03-2002
			EP 1315511 A2	04-06-2003
			HU 0303854 A2	01-03-2004
			JP 2004515471 T	27-05-2004
			NO 20031049 A	08-05-2003
			SK 2772003 A3	07-10-2003
			AU 7338501 A	22-03-2002
			AU 7338801 A	22-03-2002
			AU 8893701 A	22-03-2002
			BR 0113579 A	17-06-2003
			CA 2412278 A1	14-03-2002
			CA 2412279 A1	14-03-2002
			CA 2412298 A1	14-03-2002
			CN 1457257 T	19-11-2003
			CN 1452632 T	29-10-2003
			CN 1454097 T	05-11-2003
			CN 1458846 T	26-11-2003
			CZ 20030678 A3	17-03-2004
			EP 1318827 A1	18-06-2003
			EP 1315513 A2	04-06-2003
			EP 1315738 A1	04-06-2003
			JP 2004508338 T	18-03-2004
			JP 2004518621 T	24-06-2004
			JP 2004508383 T	18-03-2004
			NO 20031047 A	08-05-2003
			NO 20031048 A	08-05-2003
			WO 0219963 A2	14-03-2002
			WO 0220033 A1	14-03-2002
			WO 0220034 A1	14-03-2002
			WO 0220557 A1	14-03-2002
			US 2003208046 A1	06-11-2003
			ZA 200300314 A	04-02-2004
			ZA 200300315 A	04-02-2004
			ZA 200300659 A	05-03-2004
WO 2004009082	A	29-01-2004	WO 2004009082 A1	29-01-2004
EP 1398328	A	17-03-2004	EP 1400533 A1	24-03-2004

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP2004/008820

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1398328 A		EP 1398328 A1	17-03-2004
		DE 02020425 T1	15-07-2004
		DE 03020423 T1	15-07-2004
		DE 03020424 T1	15-07-2004
		DE 03020425 T1	15-07-2004
		WO 2004024776 A1	25-03-2004
		WO 2004024761 A1	25-03-2004
		WO 2004024777 A1	25-03-2004
		EP 1398322 A1	17-03-2004
		EP 1398327 A1	17-03-2004
		ES 2214166 T1	16-09-2004
		ES 2213505 T1	01-09-2004
		ES 2213506 T1	01-09-2004
		ES 2213507 T1	01-09-2004
WO 02080979 A	17-10-2002	DE 10112825 A1	02-10-2002
		BG 108274 A	31-08-2004
		BR 0208126 A	02-03-2004
		CA 2441442 A1	17-10-2002
		CZ 20032430 A3	12-11-2003
		WO 02080979 A2	17-10-2002
		EP 1372735 A2	02-01-2004
		HU 0303511 A2	28-01-2004
		JP 2004525170 T	19-08-2004
		NO 20034095 A	04-11-2003
		NZ 528251 A	24-12-2004
WO 9506058 A	02-03-1995	AT 197589 T	15-12-2000
		DE 69426300 D1	21-12-2000
		DE 69426300 T2	12-04-2001
		EP 1026171 A1	09-08-2000
		EP 0714402 A1	05-06-1996
		ES 2151558 T3	01-01-2001
		WO 9506058 A1	02-03-1995
		JP 9504515 T	06-05-1997
WO 9640662 A	19-12-1996	WO 9640662 A2	19-12-1996
		US 5880270 A	09-03-1999
GB 1419080 A	24-12-1975	AU 472739 B2	03-06-1976
		AU 6377373 A	19-06-1975
		BE 809158 A1	16-04-1974
		DE 2364795 A1	04-07-1974
		FR 2212328 A1	26-07-1974
		IT 1048295 B	20-11-1980
		JP 49094618 A	09-09-1974
		LU 69055 A1	22-02-1974
		NL 7317752 A	02-07-1974
		US 3941777 A	02-03-1976
		ZA 7309540 A	27-11-1974
EP 0243929 A2	04-11-1987	CA 1341053 C	18-07-2000
		DE 3751537 D1	02-11-1995
		DE 3751537 T2	28-03-1996
		JP 2900992 B2	02-06-1999
		JP 8259600 A	08-10-1996
		JP 2528464 B2	28-08-1996
		JP 62267300 A	19-11-1987

INTERNATIONAL SEARCH REPORT

tion on patent family members

International Application No
PCT/EP2004/008820

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0243929	A2	US 6673347 B1	06-01-2004
		US 2004081660 A1	29-04-2004
<hr/>			